39. Katims JJ, Naviasky EH, Ng LK, Rendell M, Bleecker ML. New screening device for assessment of peripheral neuropathy. J Occup Med 1986;28: 1219–1221
40. Masson EA, Veves A, Fernando D, Boulton AJ. Current perception thresholds: a new, quick, and reproducible method for the assessment of peripheral neuropathy in diabetes mellitus. Diabetologia 1989;32:724–728
41. Veves A, Young MJ, Manes C, Boulton AJ. Differences in peripheral and autonomic nerve function measurements in painful and painless neuropathy: a clinical study. Diabetes Care 1994;17:1200–1202
42. Matsutomo R, Takebayashi K, Aso Y. Assessment of peripheral neuropathy using measurement of the current perception threshold with the neurometer in patients with type 2 diabetes mellitus. J Int Med Res 2005;33:442–453
43. Yamamoto H, Lee CE, Marcus JN, et al. Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons. J Clin Invest 2002;110:43–52
44. Barragán JM, Eng J, Rodríguez R, Blázquez E. Neural contribution to the effect of glucagon-like peptide-1-(7-36) amide on arterial blood pressure in rats. Am J Physiol 1999;277:E784–E791
45. Li Y, Hansotia T, Yusta B, Ris F, Halban PA, Drucker DJ. Glucagon-like peptide-1 receptor signaling modulates beta cell apoptosis. J Biol Chem 2003;278:471–478
46. Maida A, Hansotia T, Longuet C, Seino Y, Drucker DJ. Differential importance of glucose-dependent insulinotropic polypeptide vs glucagon-like peptide 1 receptor signaling for beta cell survival in mice. Gastroenterology 2009;137:2146–2157
47. Miki T, Liss B, Minami K, et al. ATP-sensitive K+ channels in the hypothalamus are essential for the maintenance of glucose homeostasis. Nat Neurosci 2001;4:507–512
48. Burcelin R, Knauf C, Cani PD. Pancreatic alpha-cell dysfunction in diabetes. Diabetes Metab 2008;34(Suppl. 2):S49–S55

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A comparative analysis of intraperitoneal versus intracerebroventricular administration of bromodeoxyuridine for the study of cell proliferation in the adult rat brain

M. Cifuentes[a], M. Pérez-Martín[a], J.M. Grondona[a], M.D. López-Ávalos[a], N. Inagaki[c], P. Granados-Durán[a], P. Rivera[b], P. Fernández-Llebrez[a,*]

[a] Departamento de Biología Celular, Genética y Fisiología, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain
[b] Laboratorio de Medicina Regenerativa, Hospital Carlos Haya, Fundación IMABIS, Avda. Carlos Haya 82, Pabellón de Gobierno, 29010 Málaga, Spain
[c] Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT

Bromodeoxyuridine (BrdU) is the most widely used marker to detect proliferative cells in the adult brain. Here we analyse whether the route of administration of the tracer influences the number of labelled cells. For the intraperitoneal (ip) administration of BrdU, we performed two daily injections during 7 days, and for an intracerebroventricular (icv) delivery, it was continuously infused into one lateral ventricle for a 7 days period as well. After ip administration, cells labelled with BrdU were seen in the subventricular zone of the striatal wall of the lateral ventricle, the hippocampus and the neurohemal circumventricular organs. Also, the habenula and large myelinated tracts, such as the fornix and the corpus callosum, showed many BrdU-positive nuclei. Labelled nuclei were scarce in the parenchymal regions of the rest of the brain. In contrast, a significant increase in the number of BrdU-positive nuclei was observed in the parenchyma of the periventricular zones after icv administration of the marker, thus showing a greater availability of the tracer when it was administered directly into the ventricular cerebrospinal fluid. We suggest that the availability of BrdU in the vicinity of proliferating cells may depend on the permeability of the brain vessels to nucleosides in each location. By using double immunocytochemistry we found that neurons, astrocytes, oligodendrocytes, tanycytes and microglia had incorporated the tracer, demonstrating their proliferation capacity.

1. Introduction

5-Bromo-2′-deoxyuridine (BrdU) is the most widely used marker to study cell proliferation in the adult brain (Kempermann, 2006). It is incorporated into the newly synthesized DNA during the S phase in the place of deoxythymidine, and can be easily detected using specific antibodies. After administration of BrdU, it is assumed that the number of labelled nuclei reflects the intensity of cell proliferation at a given place. However, the availability of BrdU greatly depends on the administration protocol, that is, the route of delivery, the scheduled timing, the permeability of the barriers found between the compartment of delivery and the location of the cells under study, and the potential clearance capacity of the tissue for BrdU (Spector and Johanson, 2007).

In most studies on cell proliferation in the adult brain, BrdU was administered peripherally by intraperitoneal (ip) or intravascular injection, or even in the drinking water. In these cases it was assumed that BrdU enters the brain through the blood–brain barrier (BBB) or the blood–cerebrospinal fluid (CSF) barrier (i.e. the ependymal cells of the choroid plexus). Equilibrative nucleoside transporters located in the endothelial cells of the brain vessels and in the ependymal cells of the choroid plexuses, make possible the transport of BrdU from the blood to the intercellular spaces of the brain parenchyma by facilitated diffusion (Redzic et al., 2005; Spector and Johanson, 2007). On the other hand, concentrative nucleoside transporters, located in the abluminal membrane of the endothelial cells of the brain vessels and in the apical membrane of the ependymal cells of the choroid plexuses, would be responsible for the rapid clearance of nucleosides from the intercellular spaces of the brain and from the ventricular CSF by active transport (Redzic et al., 2005; Spector and Johanson, 2007). However, it is not known

Abbreviations: AP, area postrema; BBB, blood–brain barrier; BrdU, 5-bromo-2′-deoxyuridine; CNS, central nervous system; CSF, cerebrospinal fluid; CVO, circumventricular organ; GFAP, glial fibrillary acidic protein; ip, intraperitoneal; icv, intracerebroventricular; ME, median eminence; OVLT, organon vasculosum of the lamina terminalis; SCO, subcommissural organ; SFO, subfornical organ; SGZ, subgranular zone; SVZ, subventricular zone.

* Corresponding author. Tel.: +34 9 52131858; fax: +34 9 52132000.
E-mail address: llebrez@uma.es (P. Fernández-Llebrez).

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whether these nucleoside transport systems operate similarly in different brain zones and, consequently, if the BrdU administered in the periphery is equally available in all regions of the adult brain.

Neurogenesis in the central nervous system (CNS) of adult rodents is prominent in two main locations: the subventricular zone (SVZ) of the striatal wall of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Ming and Song, 2005). More recently it was shown that the circumventricular organs contain neural stem cells that give rise to neurons and glia (Bennett et al., 2009, 2010). To a lower extent, cell proliferation was reported as well in many other periventricular regions of the brain and the spinal cord of adult rodents, sometimes after the stimulation with growth factors (Pencea et al., 2001; Chouaf-Lakhdar et al., 2003; Xu et al., 2005; Danilov et al., 2006; Kokoeva et al., 2005, 2007; Pérez-Martín et al., 2010). In an interesting study, Kokoeva et al. (2007) showed that the number of BrdU-labelled cells in the hypothalamus of mice dramatically increased when BrdU was administered through a cannula inserted into one lateral ventricle compared to animals that were injected ip.

The aim of the present investigation was to study cell proliferation in different regions of the brain of the adult rat after BrdU administration either ip or intracerebroventricularly (icv). We compared the proliferation observed in the highly permeable neurohemal regions with that in other zones with a well-developed BBB. We also evaluated the nature of the newborn cells, and showed that neurons, astrocytes, tanycytes, oligodendrocytes and microglia are constitutively generated in several regions of the adult rat brain.

2. Materials and methods

2.1. Animals

Adult male Wistar rats ($n = 10$) weighting about 200 g (Charles River Laboratories, Wilmington, MA) were used in this study. Animals were kept under a photoperiod of 12L:12D with free access to food and water. Manipulation of the animals followed the principles of laboratory animal care published by Spanish Ethical Committee (RD 1201/2005) and the European Union normative (86/609/EEC).

2.2. Implantation of the intracerebroventricular cannula

The rats were anaesthetized with 2,2,2-tribromoethanol (300 mg/kg body weight). An osmotic minipump (flow rate 1 μl/h; Alzet 2001, Alza, Palo Alto, CA, USA) was subcutaneously implanted between the scapulae, and maintained for 7 days. The minipump was attached to a brain infusion cannula (Alza) through polyethylene tubing and primed by immersion in normal saline at 40 °C for 4 h. These procedures were conducted under sterile conditions. The cannula was implanted into the right lateral cerebral ventricle (−0.5 mm anteroposterior, −1.4 mm lateral and −3.3 mm dorsoventral; coordinates based on Paxinos and Watson, 1986) and secured to the skull with dental cement. The minipumps was filled with saline or BrdU solution.

2.3. Experimental groups

2.3.1. Group 1 (G1): intracerebroventricular saline plus intraperitoneal BrdU

Four rats received saline (0.9% NaCl, 1 μl/min) for 7 days into the right lateral ventricle while BrdU (Sigma) was ip injected (10 mg/ml in 0.9% NaCl, 50 mg/kg body weight) twice per day (every 12 h, early in the morning and late in the evening) during the same 7 days. This represents a total amount of 140 mg per animal during the 7 days. Animals were sacrificed 12 h after the last BrdU injection.

2.3.2. Group 2 (G2): intracerebroventricular BrdU

Four rats were icv administered with BrdU (1 mg/ml in 0.9% NaCl) continuously infused for 7 days through a cannula as described above (flow rate 1 μl/h). This represents a total amount of 0.17 mg per animal during the 7 days.

2.3.3. Group 3 (G3): intraperitoneal BrdU

Two rats were ip injected with BrdU, as described above for experimental group 1, but without icv cannula implantation.

2.4. Tissue processing and immunohistochemistry

The animals were anaesthetized with 2,2,2-tribromoethanol and perfused through the aorta with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Brains were removed from the skull, immersed in the same fixative for 4 h at 4 °C, and rinsed with PB. Coronal sections (50 μm thick) were obtained with a vibratome (Leica Heidelberg, Germany).

2.4.1. BrdU immunohistochemistry

For BrdU detection we used a monoclonal mouse anti-BrdU antibody. This is an IgG_1 monoclonal antibody obtained from ascites by using BrdU-bovine serum albumin (72–73 kDa) as immunogen. Dilution: 1:5000. Source: Hybridoma Bank, Iowa City, IA, USA; Ref. G3G4. This antibody was previously used for studies on rat CNS cell proliferation (Pérez-Martín et al., 2010; Rivera et al., 2011; for more information about the antibody and references about the use of this antibody go to http://dshb.biology.uiowa.edu/G3G4AntiBrdUrd).

For immunostaining, the endogenous peroxidase activity in the tissue sections was first quenched by incubation in 3% H_2O_2 and 10% methanol in PB for 30 min at room temperature (RT). As a previous antigen retrieval step, DNA was denatured by incubating the sections in 2 N HCl for 15 min at 37 °C, and rinsed twice in 0.1 M borate buffer (pH 8.5). Blocking of non-specific binding sites was done by an incubation step with PST (0.3% Triton X-100, 0.3% bovine serum albumin in 0.1 M PB). 0.1 M PB buffer was used in all the following washes and PST for the dilution of the antibodies. Sections were incubated overnight at 4 °C in agitation in primary antibody. After rinsing, sections were incubated for 90 min with biotinylated goat anti-mouse IgG (1:1000, Pierce, Rockford, IL, USA), rinsed and transferred to the avidin–biotin peroxidase complex solution (1:250; Pierce) for 45 min. Peroxidase was detected using a solution of 0.05% diaminobenzidine (DAB, Sigma) as chromogen. In control sections incubation with the primary antibody was omitted.

2.4.2. Double immunofluorescence

Sections obtained from animals of group 2 were used for the identification of the proliferationg cells using the following antibodies: rat anti-BrdU (1:2000; Accurate Scientific, Westbury, NY, USA), mouse anti-GFAP (1:2000; Sigma) for the detection of astrocytes, mouse anti-vimentin (1:2000; Sigma) for ependymocytes, mouse anti-NeuN (1:1000, Chemicon, Temecula, CA) for neurons, rabbit polyclonal anti-Iba-1 (1:1000, Wako Chemical USA Inc., Richmond, VA, USA) for microglia; rabbit anti rat-ABCA2 (Zhou et al., 2001) for oligodendrocytes. For the detection of BrdU-labelled nuclei, DNA was denatured as mentioned above, and then the sections were incubated overnight at 4 °C with a combination of the selected primary antibodies. After washing in PB, sections were incubated for 2 h with the secondary antibodies diluted 1:1000: goat anti-rat IgG labelled with Alexa 488, donkey anti-mouse IgG Alexa 594, or donkey anti-rabbit IgG Alexa 594 (Molecular Probes). The labelled sections were visualized with a confocal microscope (Leica Microsystems). In some sections nuclei were stained with

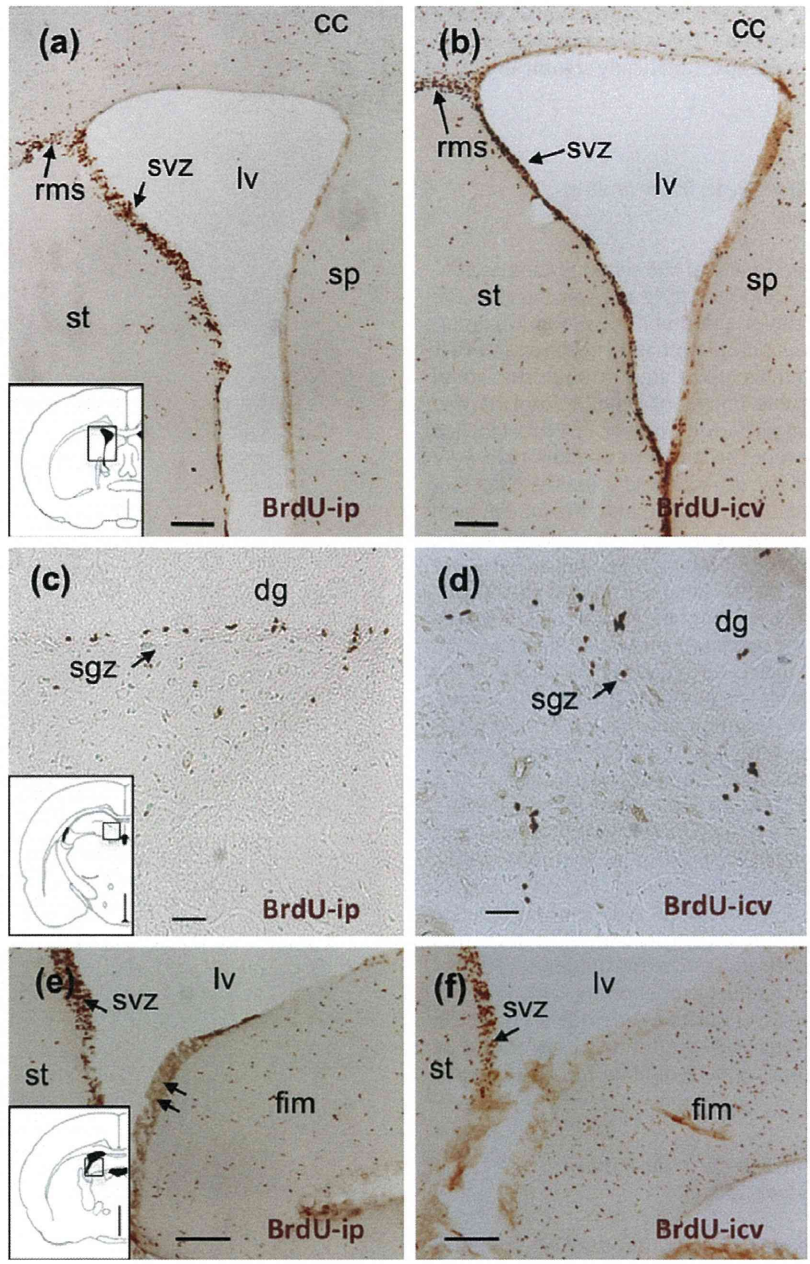

Fig. 1. (a) and (b) BrdU-positive nuclei around the lateral ventricle (LV) of rats treated either ip (BrdU-ip) or icv (BrdU-icv) with the proliferation marker BrdU. Numerous positive nuclei occupied the subventricular zone (SVZ) and the rostral migratory stream (RMS) in both cases. Note the higher amount of positive nuclei in the striatum (ST), septum (SP) and corpus callosum (CC) of the icv-injected animal. (c) and (d) BrdU-positive nuclei in the subgranular zone (SGZ) beneath the granular cell layer (GCL) of the hippocampus. (e) and (f) BrdU-positive nuclei in the fimbria (FIM). A larger amount of positive nuclei appear in the icv-treated animals. The double arrow in (e) points to ependymal cells lining the fimbria showing a BrdU-positive cytoplasm. Micrographs correspond to the framed zone at the level of each scheme. Bars: (a), (b), (e), and (f): 200 μm; (c) and (d): 20 μm.

DAPI. Control sections for the immunostaining were included omitting the incubation in the primary antibody.

2.5. Quantitative and statistical analyses

The quantitative analysis of BrdU-positive cells was performed on coded sections of the hypothalamus of animals belonging to groups 1 and 2. BrdU-positive cells were counted on every 6th section (300 μm apart) (−1.30 mm to −3.60 mm from bregma). The same areas and number of sections (seven sections) were studied for all the animals in both experimental groups. All BrdU-positive cells were counted with a 40× microscope objective. The total number of BrdU-labelled cells was estimated as described by Cameron and McKay (2001) and Kempermann et al. (1997). Briefly, BrdU-immunoreactive nuclei that came into focus while focusing down through the thickness of the section were counted, according to the optical dissector principle (West, 1993; Coggeshall and Lekan, 1996). The positive nuclei located in the uppermost focal plane were ignored. We considered BrdU-positive nuclei those showing the label in the whole surface or in patches of variable intensity. The number of BrdU-labelled cells was multiplied by 6 (because every sixth section had been used) in order to estimate the total number of BrdU-immunoreactive cells.

Data were analysed using the t-test for comparisons. Data are shown as means ± SEM. A value of $p < 0.05$ was established to consider the differences between groups statistically significant.

3. Results

3.1. Distribution of BrdU-positive nuclei in the brain of intraperitoneal injected animals

No label could be observed in any of the sections used as control for the immunostaining, demonstrating the specificity of the anti-BrdU antibodies. With the exception of the site of implantation of the cannula, there was no inflammatory reaction in other zones of the brain of the animals cannulated through the lateral ventricle. To assess the possible impact of cannula implantation on cell proliferation, we compared the number of BrdU-labelled nuclei after ip administration of the tracer in animals that were cannulated icv (saline infused icv; group 1) and those that were not cannulated (group 3). We obtained roughly similar results in both cases. For this reason, the following description is based mostly on the results seen in experimental group 1. Many BrdU-positive nuclei were seen in the SVZ of the striatal wall and in the rostral migratory stream (Fig. 1a). A moderate number were seen in the corpus callosum and some appeared dispersed in the septum (Fig. 1a). Also BrdU-positive nuclei were common in the SGZ of the hippocampal dentate gyrus (Fig. 1c). At more caudal levels, a moderate number of BrdU-positive nuclei were seen in the fimbria of the hippocampus (Fig. 1e). The diencephalon showed very few positive nuclei with the notable exception of the neurohemal organs, such as the median eminence (ME) (Fig. 2a and c) the subfornical organ (SFO) (Fig. 4a) and the organon vasculosum of the lamina terminalis (OVLT) (Fig. 4c) that showed frequent positive nuclei. Interestingly, the habenula also showed BrdU-positive nuclei (Fig. 2d). The subcommissural organ (SCO) and surroundings did not show labelled nuclei (Fig. 4e). More caudally, positive nuclei were seen in another neurohemal organ, the area postrema (AP) of the hindbrain (Fig. 4g).

In addition to the nuclear labelling proper of the BrdU incorporated to DNA during its synthesis, we also saw an evident staining in the cytoplasm of some ependymal cells (see double arrow of Fig. 1e in the ependyma of the fimbria), which could be equally observed in ip or icv injected animals.

3.2. Distribution of BrdU-positive nuclei in the brain of intracerebroventricular cannulated animals

When BrdU was administered directly into the CSF, the number of BrdU-positive nuclei was notably higher in the parenchymal regions nearby the brain ventricles up to the level of the foramen of Lukscha in the fourth ventricle (Figs. 1b, f, 2b, e and 4d, f). We quantified the number of BrdU-positive nuclei around the third ventricle. As shown in Fig. 3, the number of BrdU-positive nuclei was significantly higher in the animals in which the BrdU was administered into CSF as compared to the animals that were ip injected. Conversely, we did not found virtually any positive nuclei around the fourth ventricle in those sections caudal to the foramen of Lukscha (Fig. 4h). In these same sections, however, many BrdU-labelled nuclei were seen in peripheral parenchymal zones close to the subarachnoid space (Fig. 4h), thus suggesting that the bulk of the marker reached the outer spaces of the CSF and that it did not penetrate the caudal part of the fourth ventricle and the spinal central canal. The known neurogenic zones, such as the SVZ and the hippocampal SGZ, showed many positive nuclei, as was the case for ip-injected animals (compare Fig. 1b and d). The neurohemal organs, ME (Fig. 2b), SFO (Fig. 4b), and OVLT (Fig. 4d) and the habenula (Fig. 2e), showed a roughly similar amount of BrdU-positive nuclei in both icv-administered and ip-injected animals. Conversely, the SCO and the AP did not show positive nuclei (Fig. 4b and h).

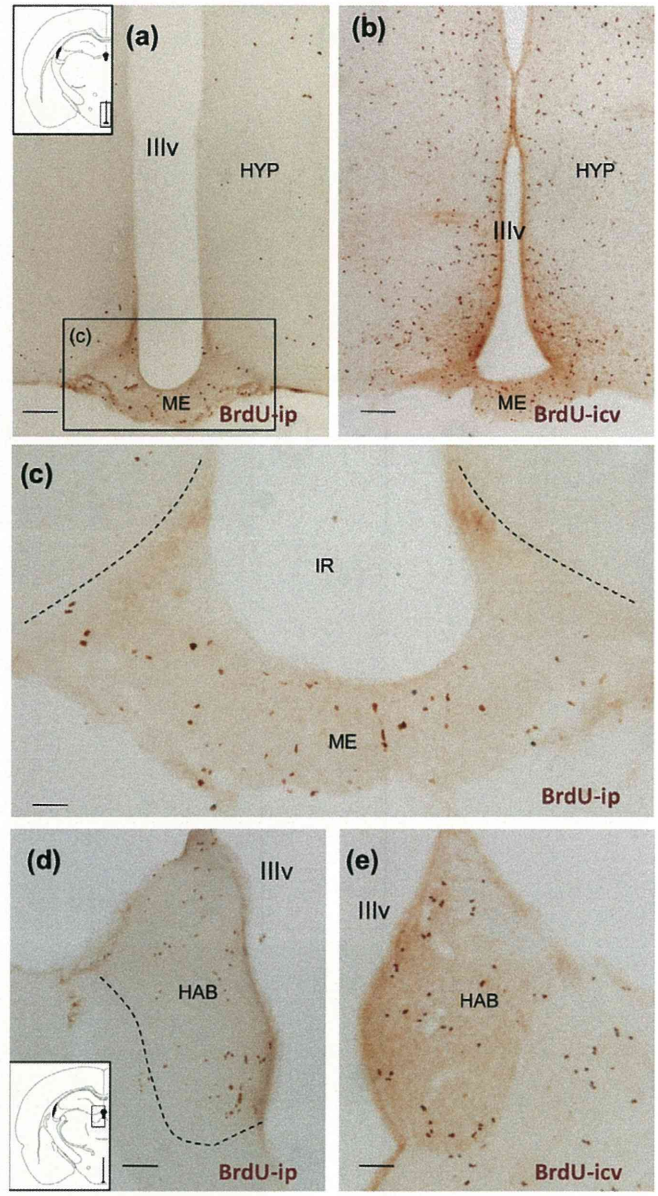

Fig. 2. (a) and (b) BrdU-positive nuclei around the hypothalamic third ventricle (IIIv) of rats injected with BrdU ip (BrdU-ip) or icv (BrdU-icv). Positive nuclei were more abundant in the hypothalamus (HYP) of icv-injected animals compared to the ip-treated ones. In these latter, positive nuclei were frequent in the median eminence (ME) (framed zone in figures (a) and (c)). (c) Detail of the ME of a BrdU-ip injected rat showing numerous BrdU-positive nuclei. The broken line indicates the limits of the neurohemal zone of the ME. IR: infundibular recess. (d) and (e) BrdU-positive nuclei in the habenular nucleus (HAB). Despite the habenula is not a neurohemal zone, the labelled nuclei are quite frequent in the BrdU-ip injected rats. The icv-injected animals present labelled nuclei in the habenula and in hypothalamic zones outside the habenula. The broken line indicates the limit of the habenular nucleus. Micrographs correspond to the framed zone at the level of each scheme. Bars: (a) and (b): 200 μm; (c): 40 μm; (d) and (e): 45 μm.

3.3. Immunocytochemical characterization of BrdU-positive cells

Double immunohistochemistry with antibodies against BrdU and different cell markers (GFAP, vimentin, NeuN, Iba1, ABCA2)

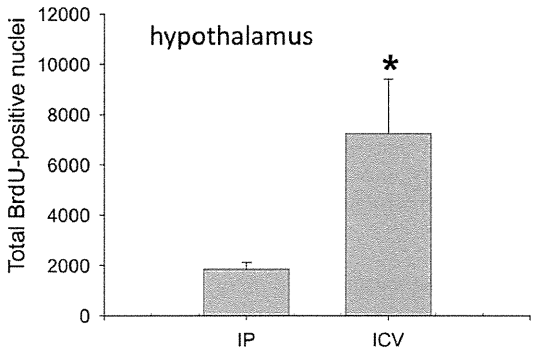

Fig. 3. Total number of BrdU-positive nuclei in the hypothalamus of rats treated with BrdU either ip (IP) or icv (ICV). The total amount of labelled nuclei is significantly higher in the icv-treated group compared to the ip-administered rats. *$p < 0.01$.

was used to study, under the confocal microscope, the nature of the BrdU-positive cells in the periventricular region, specifically in the hypothalamus and the fimbria. Animals treated with BrdU icv (experimental group 2) were used because they showed a larger amount of BrdU-positive cells in different periventricular locations. We never observed multiciliated, vimentin-positive ependymal cells labelled with BrdU. Conversely, in the ventral most portion of the third ventricle, pairs of tanycytes (positive to vimentin and GFAP) showed BrdU-positive nuclei (Fig. 5h). Some subependymal GFAP-positive astroglial cells were BrdU-positive (Fig. 5g). In the parenchymal zone, there were cell nuclei, frequently in pairs, that were positive to both NeuN and BrdU (Fig. 5a–c). Also pairs of microglial cells labelled by the Iba1 antiserum showed BrdU-positive nuclei (Fig. 5d–f). Finally, many BrdU-positive nuclei were observed in the main myelinated tracts such as the fimbria-fornix. Here, the BrdU-labelled cells were located among the oligodendrocytes that characteristically formed long rows and were stained by the antiserum against ABCA2 (Fig. 5i).

4. Discussion

4.1. The amount of BrdU-labelled nuclei depends on the availability of the tracer

In order to know the possible effect of the surgical procedure in the case of the implantation of the icv cannula, we compared results of BrdU incorporation in animals infused icv with saline and injected ip with BrdU and those treated ip with BrdU but with no icv surgery (experimental groups 1 and 3). Only the site of implantation of the cannula showed a feebly inflammatory reaction. Moreover, since the amount and localization of the proliferating cells was similar in both groups of animals, we concluded that cannula implantation did not affect the proliferative capacity and activity of the cells inside the rat CNS.

Many studies on cell proliferation in the adult CNS rely on the identification of nuclei that incorporate BrdU during the S phase of the cell cycle, as it is an easily detectable tracer. The number of cells labelled after BrdU administration depend on the ammount of BrdU available nearby the proliferating cells (Cameron and McKay, 2001). Frequently tracer studies underestimate the number of neurons and glial cells that have divided, partly because the tracer does not have free access to all locations of the brain (see, Spector and Johanson, 2007; Balu et al., 2009). To investigate the impact of the putative limitation of BrdU availability in the CNS we compared two different methodological approaches to administer the tracer into the adult CNS, that is, intraperitoneal (ip) versus intracerebroventricular (icv). We identified a higher amount of labelled cells when the tracer was administered via icv than when it was injected via ip, especially in the parenchyma.

In the present investigation, the total amount of BrdU used in ip-injected animals was one thousand times higher than the amount used in icv infused animals. However, the brain represents about 1% of the body weight, and thus about 1% of the BrdU injected in the periphery is expected to reach the CNS. Moreover, according to Hayes and Nowakowski (2000), the half-life of BrdU in blood is about 30–60 min. Hence, the BrdU injected in the periphery would be available within the brain only during 1 or 2 h a day, in contrast to the 24 h of free availability in the case of the central administration of BrdU with an osmotic minipump. Considering that the tracer incorporates into the cell nuclei in S phase, and that the length of the cell cycle of the proliferating cells in different brain regions is unknown, it is expected that more cells would take the tracer if it were available continuosly instead of for a limited period of time. Moreover, when BrdU is injected ip, both the BBB, at the wall of the brain vessels, and the blood–CSF barrier, at the ependymal cells of the choroid plexus, restrict the diffusion of nucleosides between the blood and the intercellular spaces of the brain. Conversely the ependymal cells that line the brain ventricles allow the passage of nucleosides from the ventricular CSF to the interstitial spaces of the brain when BrdU is delivered into the cerebral ventricles (Spector and Johanson, 2007). All these reasons could explain, at least in part, the results obtained in the present investigation, which are in agreement with those reported by Pencea et al. (2001) in rats, and by Kokoeva et al. (2007) in mice. As these authors, we also found a higher number of labelled nuclei when the tracer was administered through the ventricular CSF, at least in those regions that were located rostral to the foramen of Lukscha. We quantified the number of BrdU-positive cells in the periventricular area of the hypothalamus around the third ventricle, and found that there was almost a four-fold increase in the number of labelled cells when the tracer was administered via the CSF. Thus we conclude that the identification of proliferating cells within the CNS greatly depends on the local availability of the molecule used as a tracer.

Nevertheless, even when the tracer was administered peripherally, there were many labelled cells in certain regions of the brain (vg. SVZ, SGZ, habenula, fornix and the highly permeable circumventricular organs). This fact strongly suggests differences in the permeability to BrdU of the blood vessels located in different brain regions (see later).

Some authors have suggested that high doses of BrdU could result in nuclear labelling due to causes different to cell proliferation, such as DNA repair (Rakic, 2002). According to Bauer and Patterson (2005) and Kokoeva et al. (2007) it does not seem to be the case at the BrdU doses used in the present study. Still other authors reported adverse effects of long-lasting studies using BrdU that could affect the results observed (Ross et al., 2008). The possibility of cell damage has to be taken into account when evaluating proliferation using this tracer.

In the present study we observed an absence of labelled nuclei in the parenchyma around the caudal part of the fourth ventricle and the central canal when the tracer was administered through the lateral ventricle. Conversely, at the same levels, we found BrdU-positive nuclei in the brain parenchyma near the outer spaces of the CSF. In the rat, the flow of CSF along the brain ventricles is rapid (Cifuentes et al., 1992) and the bulk of CSF escapes from the fourth ventricle to the subarachnoid space through the foramen of Lucksha in the roof of the fourth ventricle (Davson et al., 1987; Grondona et al., 1996). In a similar way, the BrdU injected in the lateral ventricle probably moves quickly to the fourth ventricle (Bui et al., 1999) and escapes through the foramen of Lucksha to the outer spaces of the CSF (Jones and Sellars, 1982; Jones, 1993). Once in the outer CSF cisterns, the BrdU can penetrate the brain parenchyma through the

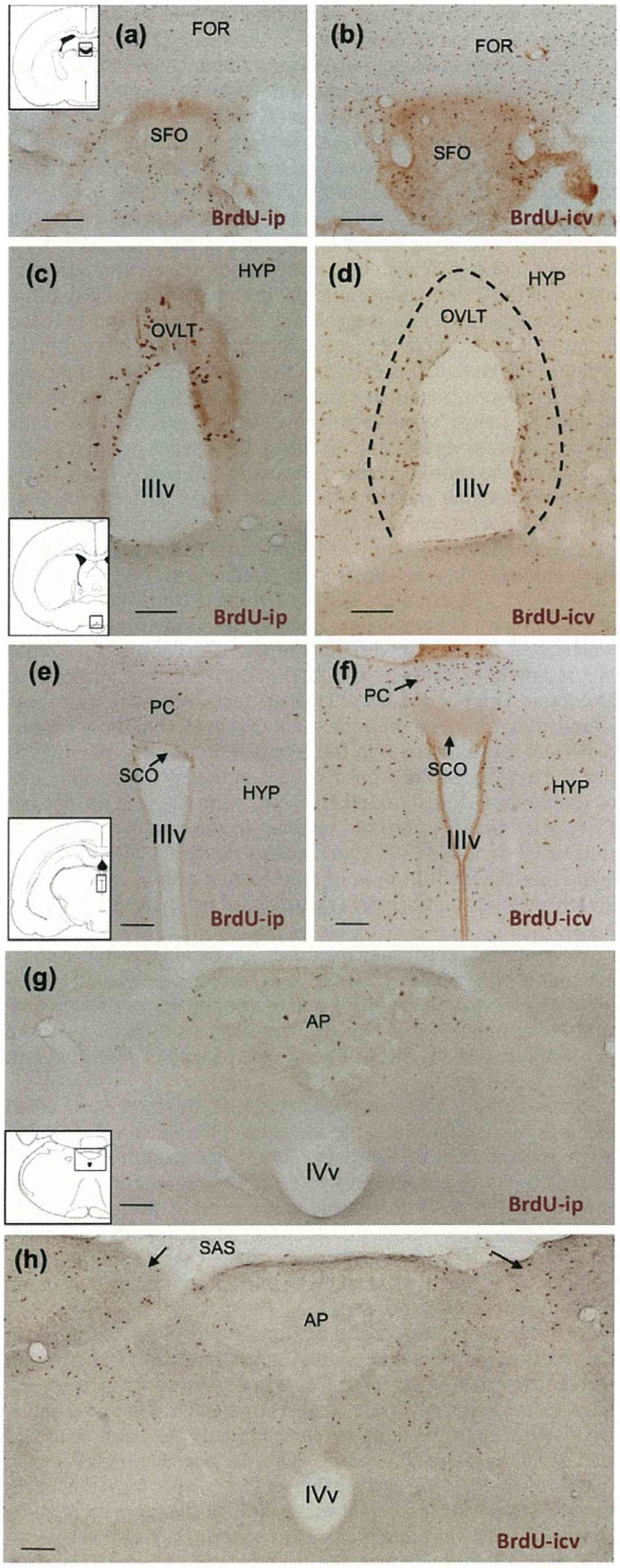

Fig. 4. (a) and (b) BrdU-positive nuclei in the subfornical organ (SFO) beneath the fornix (FOR) of rats treated ip (BrdU-ip) or icv (BrdU-icv) with BrdU. Positive nuclei are abundant in the SFO irrespective of the administration route, while in the fornix the number of positive cells is higher in icv-treated rats. (c) and (d) BrdU-positive nuclei in the organon vasculosum of the lamina terminalis (OVLT) around the rostral third ventricle (IIIv). As occur in other neurohemal organs, the ip administration results in plenty of positive cells within the OVLT, while the icv route yields abundant positive nuclei both inside the OVLT and in the surrounding hypothalamic parenchyma (HYP). The broken line indicates the limits of the OVLT. (e) and (f) BrdU-positive nuclei at the level of the subcommissural organ (SCO). Note the absence of positive nuclei in the SCO. Conversely, in the icv-administered rats BrdU-positive nuclei were abundant in the hypothalamus (HYP), including the posterior commissure (PC). (g) and (h) BrdU-positive nuclei at the level of the area postrema (AP). In ip-injected animals labelled cells appear in the AP, but are completely absent in the rombencephalic parenchyma. On the contrary, in the icv-administered animals positive nuclei were frequent in the rombencephalic parenchyma near the subarachnoid spaces (SAS) of the meninges, while the AP lacked any positive nuclei. The arrows indicate the probable route of entry of the marker from the SAS into the rombencephalic parenchyma. IVv, fourth ventricle. Micrographs correspond to the framed zone at the level of each scheme. Bars: (a) and (b): 200 μm; (c) and (d): 50 μm; (e)–(h): 200 μm.

permeable marginal glia, and hence proliferative cells in the outer margins of the brain parenchyma can be detected.

The unexpected and surprising finding that the cytoplasm of certain ependymal cells was stained with the anti-BrdU antibody will be the subject of a more detailed study in the future. Indeed we have some evidences showing that these cells display nucleoside transporters, making them candidates for the regulation of the nucleosides present in the CSF (Fernández-Llebrez, unpublished results).

4.2. Differential permeability to BrdU of the brain capillaries

Here we demonstrate the existence of dividing cells in all parts of the CNS by means of the icv-administration of BrdU. However, if the BrdU is administered ip we can detect dividing cells only in some discrete brain regions. It has been reported that the continuous capillaries of the brain (with BBB) are endowed with equilibrative nucleoside transporters that make possible the passage of BrdU (and other nucleosides) from the blood compartment to the brain (Redzic et al., 2005; Spector and Johanson, 2007). If the capillaries in all brain regions were similar, and if they all had a similar number (or type) of equilibrative nucleoside transporters, one would expect to find labelled cells in all regions of the adult CNS when BrdU is peripherally administered.

Our results, however, reveal a heterogeneous distribution of BrdU-positive nuclei when the tracer is ip-administered. Very few dividing cells were found in parenchymal zones of the CNS, such as the hypothalamus, while the number of labelled cells was high in other areas such as the SVZ of the striatal wall, the SGZ of the hippocampal gyrus dentate and, to a lower extent, the habenula, and myelinated tracts such as fimbria-fornix and corpus callosum. Besides the already known high proliferation rates at some of these locations (vg. the SVZ and the SGZ), it is likely that the permeability of the vessels to BrdU is also higher in these areas compared to other zones, such as the hypothalamus, where the number of BrdU-positive cells is scarce after ip-administration of the tracer. So far we ignore whether these differences do actually exist and what is the anatomical basis for them. In the particular case of the habenula, some authors have described leaky junctions in the local blood vessels that make them more permeable to solutes (Zhuang et al., 1996).

4.3. Labelling of nuclei in neurohemal organs after intraperitoneal or intracerebroventricular administration of BrdU

When the BrdU was administered peripherally, we found many labelled nuclei within the neurohemal circumventricular organs SFO, OVLT, ME and AP, but not in the surrouding tissue. This was an expected observation, since it is known that all these organs harbour neural stem cells (Bennett et al., 2009, 2010) as well as

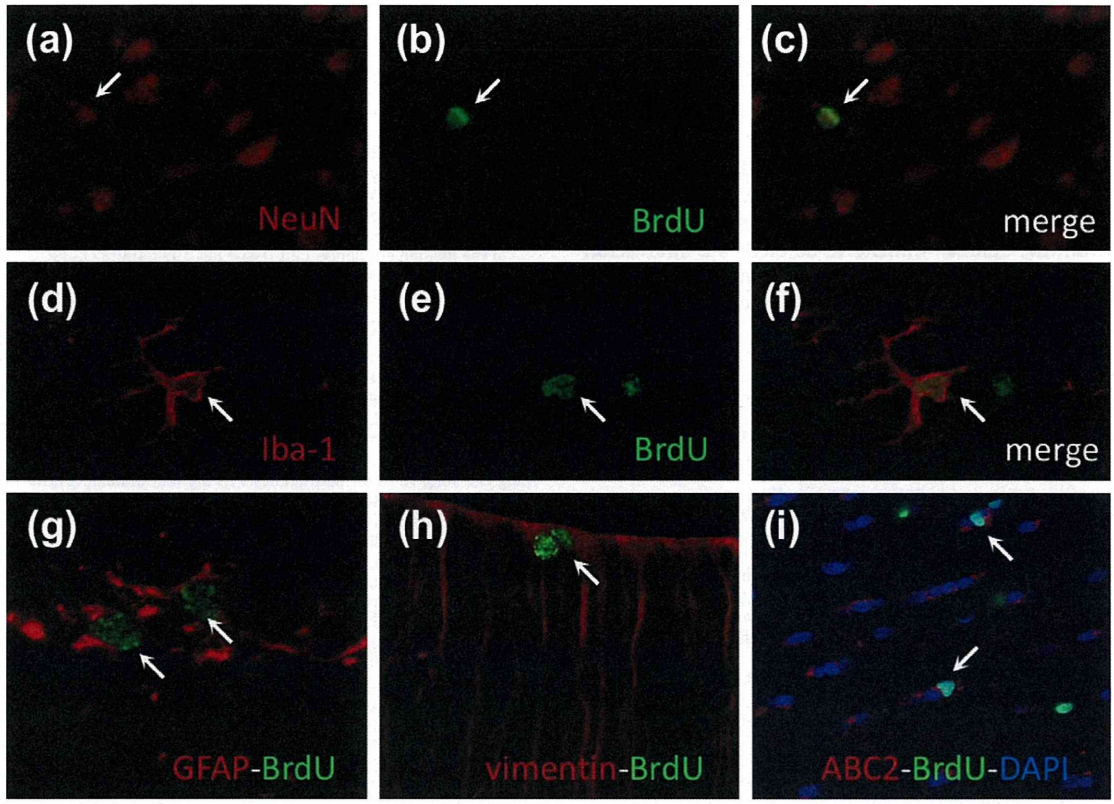

Fig. 5. Identification of the BrdU-labelled cells by double immunocytochemistry, in animals treated icv with BrdU. (a)–(c) Some neurons double labelled with NeuN and BrdU can be found in the parenchyma of the hypothalamus. (d)–(f) Iba1-imunoreactive BrdU-positive microglia appear also in the parenchyma of the hypothalamus. (g) GFAP-imunoreactive and BrdU-positive pairs of cells in the subependyma of the third ventricle. (h) A pair of BrdU-positive nuclei among vimentin-positive tanycytes of the ventral third ventricle. (i) In the fimbria, BrdU-positive cells located among the rows of ABCA2-positive oligodendrocytes. Nuclei were counterstained with DAPI. Bars: (a)–(c): 20 μm; (d)–(f): 20 μm; (g): 10 μm; (h): 20 μm; (i): 30 μm.

fenestrated capillaries (Ganong, 2000). So, the proliferating cells in the circumventricular organs have free access to the vascular BrdU. Outside the neurohemal organs, the diffusion of BrdU towards the neighbouring neuropil is restricted by the presence of glial barriers (Krisch and Leonhardt, 1978; Krisch et al., 1978, 1983, 1987), which limit the availability of BrdU. This could explain the absence of labelled nuclei in the surroundings of the neurohemal organs when the tracer was peripherally administered. Interestingly, we also found labelled cells inside other neurohemal organs: the ME, the SFO and the OVLT (but not the AP) when the BrdU was administered via the CSF. A possibility is that the tracer could reach the parenchyma of these circumventricular organs from the ventricular CSF by means of an intracellular transport through the ependymal cells, since the diffusion through the intercellular spaces is not possible due to the existence of glial barriers. In this respect, transependymal transport of bioactive substances from the ventricular CSF was reported in the ME of the rat (Scott and Krobisch-Dudley, 1975). Conversely to other circumventricular organs, the AP did not show any labelled nuclei when the tracer was administered via the cerebral ventricles, which could indicate that such hypothetical transependymal transport of BrdU does not exist in the AP.

4.4. Neural and non-neural cells proliferate constitutively in the adult rat CNS

Our results using double labelling of BrdU-positive cells have shown that in the nervous system of adult rats neurons, astrocytes, oligodendrocytes, tanycytes and microglia are being constitutively produced. Conversely, BrdU-positive ependymal cells were not found in any tissue section analysed. Many BrdU-stained cells expressed the neuronal marker NeuN and thus can be regarded as new neurons that arose from previously proliferating cells. In the SVZ and the SGZ, neurons represent the majority of the newly produced cells (Cameron and McKay, 2001; Alvarez-Buylla and Garcia-Verdugo, 2002). Also in the hypothalamus and other locations of the CNS, new neurons are being produced throughout the adulthood (Pencea et al., 2001; Chouaf-Lakhdar et al., 2003; Xu et al., 2005; Danilov et al., 2006; Kokoeva et al., 2005, 2007; Pérez-Martín et al., 2010). Our results show that, along with the neurons, other cell types are also produced. Several authors have reported proliferation of subependymal astrocytes and tanycytes of the third ventricle (Rodríguez et al., 2005; Xu et al., 2005; Pérez-Martín et al., 2010). Tanycytes are considered as neural stem cells in the third ventricle wall of rodents (Rodríguez et al., 2005; Xu et al., 2005). Moreover, some subependymal astrocytes located there showed similarities to SVZ neural stem cells (Pérez-Martín et al., 2010). At variance to the results of Kokoeva et al. (2007) in the mouse, we found some cells double labelled with BrdU and GFAP, which could be considered to be conventional astrocytes.

An interesting finding was the presence of numerous BrdU-positive nuclei in myelinated tracts of the CNS, especially in the fornix-fimbria. Proliferating cells were frequently located between two ABCA2-positive oligodendrocytes. Oligodendrocytes form long rows of cells in this tract (Suzuki and Raisman, 1994) and probably the newly formed cells located within these rows are oligodendrocytes as well, although the possibility that they are astrocytes cannot be excluded.

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References

Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. J Neurosci 2002;22:629–34.
Balu DT, Hodes GE, Hill TE, Ho N, Rahman Z, Bender CN, et al. Flow cytometric analysis of BrdU incorporation as a high-throughput method for measuring adult neurogenesis in the mouse. J Pharmacol Toxicol Methods 2009;59:100–7.
Bauer S, Patterson PH. The cell cycle-apoptosis connection revisited in the adult brain. J Cell Biol 2005;171:641–50.
Bennett L, Yang M, Enikolopov G, Iacovitti L. Circumventricular organs: a novel site of neural stem cells in the adult brain. Mol Cell Neurosci 2009;41:337–47.
Bennett LB, Cai J, Enikolopov G, Iacovitti L. Heterotopically transplanted CVO neural stem cells generate neurons and migrate with SVZ cells in the adult mouse brain. Neurosci Lett 2010;475:1–6.
Bui JD, Nammari DR, Buckley DL, Inglis BA, Silver XS, Mareci TH, et al. In vivo dynamics and distribution of intracerebroventricularly administered gadodiamide, visualized by magnetic resonance imaging. Neuroscience 1999;90:1115–22.
Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. J Comp Neurol 2001;435:406–17.
Cifuentes M, Fernández-Llebrez P, Pérez J, Pérez-Fígares JM, Rodríguez EM. Distribution of intraventricularly injected horseradish peroxidase in cerebrospinal fluid compartments of the rat spinal cord. Cell Tissue Res 1992;270:485–94.
Chouaf-Lakhdar L, Fèvre-Montange M, Brisson C, Strazielle N, Gamrani H, Didier-Bazès M. Proliferative activity and nestin expression in periventricular cells of the adult rat brain. Neuroreport 2003;14:633–6.
Coggeshall RE, Lekan HA. Methods for determining numbers of cells and synapses: a case for more uniform standard of rewiew. J Comp Neurol 1996;364:6–15.
Danilov AI, Covacu R, Moe MC, Langmoen IA, Johansson CB, Olsson T, et al. Neurogenesis in the adult spinal cord in an experimental model of multiple sclerosis. Eur J Neurosci 2006;23:394–400.
Davson H, Welch K, Segal MB. The physiology and pathophysiology of the cerebrospinal fluid. Edinburgh, London, Melbourne, New York: Churchill Livigstone; 1987.
Fernández-Llebrez P, Cifuentes M, Grondona JM, Pérez-Martín M, López-Ávalos MD. BrdU is present in the cytoplasms of the ependymal cells of intraperitoneal and intracerebroventricular BrdU-injected rats. Unpublished results; 2011.
Ganong WF. Circumventricular organs: definition and role in the regulation of endocrine and autonomic function. Clin Exp Pharmacol Physiol 2000;27:422–7.
Grondona JM, Pérez-Martín M, Cifuentes M, Pérez J, Jiménez AJ, Pérez-Fígares JM, et al. Ependymal denudation, aqueductal obliteration and hydrocephalus after a single injection of neuraminidase into the lateral ventricle of adult rats. J Neuropathol Exp Neurol 1996;55:999–1008.
Hayes NL, Nowakowski RS. Exploiting the dynamics of S-phase tracers in developing brain: interkinetic nuclear migration for cells entering versus leaving the S-phase. Dev Neurosci 2000;22:44–55.
Jones HC, Sellars RA. The movement of fluid out of the cerebral ventricles in the fetal and neonatal rat. Z Kinderchir 1982;37:130–3.
Jones HC. Physiology of cerebrospinal fluid circulation: amphibians, mammals and hydrocephalus. In: Oksche A, Rodríguez EM, Fernández-Llebrez P, editors. The subcommissural organ an ependymal brain gland. Berlin, Heidelberg: Springer-Verlag; 1993. p. 243–53.
Kempermann G. Adult neurogenesis. New York: Oxford; 2006.
Kempermann G, Kuhn HG, Gage FH. Genetic influence of neurogenesis in the dentate gyrus of adult mice. PNAS 1997;94:10409–14.
Kokoeva MV, Yin H, Flier JS. Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science 2005;310:679–83.
Kokoeva MV, Yin H, Flier JS. Evidence for constitutive neural cell proliferation in the adult murine hypothalamus. J Comp Neurol 2007;505:209–20.
Krisch B, Leonhardt H. The functional and structural border of the neurohemal region of the median eminence. Cell Tissue Res 1978;192:327–39.
Krisch B, Leonhardt H, Buchheim W. The functional and structural border between the CSF- and blood-milieu in the circumventricular organs (organum vasculosum laminae terminalis, subfornical organ, area postrema) of the rat. Cell Tissue Res 1978;195:485–97.
Krisch B, Leonhardt H, Oksche A. The meningeal compartments of the median eminence and the cortex. A comparative analysis in the rat. Cell Tissue Res 1983;228:597–640.
Krisch B, Leonhardt H, Oksche A. Compartments in the organum vasculosum laminae terminalis of the rat and their delineation against the outer cerebrospinal fluid-containing space. Cell Tissue Res 1987;250:331–47.
Ming GL, Song H. Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 2005;28:223–50.
Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2nd ed. Sydney: Academic Press; 1986.
Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 2001;17:6706–17.
Pérez-Martín M, Cifuentes M, Grondona JM, López-Avalos MD, Gómez-Pinedo U, García-Verdugo JM, et al. IGF-I stimulates neurogenesis in the hypothalamus of adult rats. Eur J Neurosci 2010;31:1533–48.
Rakic P. Adult neurogenesis in mammals: an identity crisis. J Neurosci 2002;22:614–8.
Redzic ZB, Biringer J, Barnes K, Baldwin SA, Al-Sarraf H, Nicola PA, et al. Polarized distribution of nucleoside transporters in rat brain endothelial and choroid plexus epithelial cells. J Neurochem 2005;94:1420–6.
Rivera P, Romero-Zerbo Y, Pavón FJ, Serrano A, López-Ávalos MD, Cifuentes M, et al. Obesity-dependent cannabinoid modulation of proliferation in adult neurogenic regions. Eur J Neurosci 2011;33:1577–86.
Rodríguez EM, Blázquez JL, Pastor FE, Peláez B, Peña P, Peruzzo B, et al. Hypothalamic tanycytes: a key component of brain–endocrine interaction. Int Rev Cytol 2005;247:89–164.
Ross HH, Levkoff LH, Marshall 2nd GP, Caldeira M, Steindler DA, Reynolds BA, et al. Bromodeoxyuridine induces senescence in neural stem and progenitor cells. Stem Cells 2008;26:3218–27.
Scott DE, Krobisch-Dudley G. Ultrastructural analysis of the mammalian median eminence. Morphological correlate of transependymal transport. In: Knigge KM, Scott DE, Weindl A, editors. Brain–endocrine interaction II. The ventricular system in neuroendocrine mechanisms. Basel: S Karger; 1975. p. 29–39.
Spector R, Johanson CE. The origin of deoxynucleosides in brain: implications for the stury of neurogenesis and stem cell therapy. Pharm Res 2007;24:859–67.
Suzuki M, Raisman G. Multifocal pattern of postnatal development of the macroglial framework of the rat fimbria. Glia 1994;12:294–308.
West MJ. Regionally specific loss of neurons in the aging human hippocampus. Neurobiol Aging 1993;14:287–93.
Xu Y, Tamamaki N, Noda T, Kimura K, Itokazu Y, Matsumoto N, et al. Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol 2005;192:251–64.
Zhou C, Zhao L, Inagaki N, Guan J, Nakajo S, Hirabayashi T, et al. ATP-binding cassette transporter ABC2/ABCA2 in the rat brain: a novel mammalian lysosome-associated membrane protein and a specific marker for oligodendrocytes but not for myelin sheaths. J Neurosci 2001;21:849–57.
Zhuang X, Silverman AJ, Silver R. Brain mast cell degranulation regulates blood–brain barrier. J Neurobiol 1996;31:393–403.

Randomized controlled trial of single-agent glimepiride and pioglitazone in Japanese patients with type 2 diabetes: A comparative study

Nobuyuki Shihara[1], Masafumi Kitaoka[1,2], Nobuya Inagaki[1,3], Takashi Kadowaki[1,4], Seisuke Koumoto[1], Jo Satoh[1,5], Yasuo Terauchi[1,6], Kiyohide Nunoi[1,7], Yuichiro Yamada[1,8], Hiroyuki Sakamaki[9], Yutaka Seino[1,10]*

ABSTRACT

Aims/Introduction: To compare first-line, single-agent glimepiride and pioglitazone in Japanese patients with type 2 diabetes uncontrolled by diet and exercise with respect to glycemic control, safety and metabolic changes.

Materials and Methods: Patients with previously untreated type 2 diabetes were enrolled in a multicenter, randomized, non-blind, parallel-group trial of glimepiride (0.5–6 mg/day) or pioglitazone (15–45 mg/day) for 6 months.

Results: A total of 191 patients aged 30–75 years were randomized. Similar percentages of patients attained the primary end-point, with glycated hemoglobin < 6.9% at month 6 with glimepiride and pioglitazone, respectively (61.2 vs 56.8%, $P = 0.64$). At month 6, the following significant ($P < 0.05$) intragroup changes in mean plasma lipid concentrations were noted as compared with baseline: total cholesterol decreased from 203.5 to 195.5 mg/dL and low-density lipoprotein (LDL)-cholesterol decreased from 124.5 to 116.3 mg/dL in the glimepiride group, whereas high-density lipoprotein (HDL)-cholesterol increased from 51.6 to 56.0 mg/dL and triglycerides decreased from 167.6 to 143.6 mg/dL in the pioglitazone group. The only symptomatic adverse events were mild-to-moderate in four patients receiving pioglitazone, and constipation in one patient receiving glimepiride. Similar numbers of patients experienced asymptomatic hypoglycemia (<60 mg/dL) in the glimepiride and pioglitazone groups ($n = 7$ and 5, respectively).

Conclusions: There was no statistically significant difference between glimepiride and pioglitazone with respect to glycemic control, and both agents were well tolerated. Glimepiride significantly lowered total cholesterol and LDL-cholesterol, whereas pioglitazone increased HDL-cholesterol. This trial was registered with University Hospital Medical Information Network (UMIN), Japan, UMIN000004582. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2011.00115.x, 2011)

KEY WORDS: Glimepiride, Pioglitazone, Type 2 diabetes mellitus

INTRODUCTION

Consistent with other developed countries, there has been a steady increase in Japan in the number of patients with diabetes. According to the 2007 National Health and Nutrition Survey carried out by the Ministry of Health, Labour and Welfare, the estimated number of persons in Japan strongly suspected of having diabetes (includes those on diabetes treatment) increased to approximately 8.9 million as compared with 6.9 million in 1997 and 7.4 million in 2002[1].

Sulfonylureas are the most frequently used first-line oral anti-diabetic drug class in Japan; they are well suited to the predominant etiology of Japanese diabetic patients; that is, impaired insulin secretion[2]. Long-term follow up (10 years) of patients with type 2 diabetes mellitus enrolled in the United Kingdom Prospective Diabetes Study (UKPDS) showed that, compared with dietary therapy alone, those treated with intensive therapy with either a sulfonylurea or insulin had a significantly reduced relative risk of microvascular disease (24%, $P = 0.001$), myocardial infarction (15%, $P = 0.01$), diabetes-related death (17%, $P = 0.01$) or death from any cause (13%, $P = 0.007$); these benefits were attained despite between-group differences in glycated hemoglobin (HbA_{1c}) being lost after the first year[3]. Although sulfonylureas confer reliable glycemic control, there has been some concern over the risk of hypoglycemia and weight gain[4]. However, the third-generation sulfonylurea, glimepiride, has a relatively low risk of hypoglycemia[5].

[1]Japan Association for Diabetes Education and Care, [2]Division of Endocrinology and Metabolism, Showa General Hospital, [4]Department of Diabetes and Metabolic Diseases, The University of Tokyo, Tokyo, [3]Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, [5]Division of Diabetes and Metabolism, Department of Internal Medicine, Iwate Medical University School of Medicine, Iwate, [6]Department of Endocrinology and Metabolism, Yokohama City University Graduate School of Medicine, Kanagawa, [7]Division of Endocrinology and Metabolism, St Mary's Hospital, Fukuoka, [8]Department of Endocrinology, Diabetes and Geriatric Medicine, Akita University School of Medicine, Akita, [9]Department of Clinical Economics, Faculty of Pharmaceutical Science, Meijo University, Aichi, and [10]Kansai Electric Power Hospital, Osaka, Japan

*Corresponding author. Yutaka Seino Tel.: +81-3-3514-1721 Fax: +81-3-3514-1725
E-mail address: seino.yutaka@e2.kepco.co.jp

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Pioglitazone improves insulin resistance and is the only approved thiazolidinedione in Japan. It is commonly used as a first-line treatment for patients with type 2 diabetes mellitus in Japan. Pioglitazone was expected to reduce cardiovascular event risk[6]. However, concern was raised when another thiazolidinedione, rosiglitazone, was shown to significantly increase the risk of myocardial ischemic events compared with placebo in patients with type 2 diabetes mellitus[7]. A meta-analysis of randomized trials showed that pioglitazone reduced the risk of myocardial ischemic events, although it increased the risk of serious heart failure[8]. In addition to the increase in heart failure, several adverse events have received attention during treatment with pioglitazone, including weight gain, edema[9] and fracture[10].

Various randomized trials have been published that compare glimepiride and pioglitazone in Western patients with type 2 diabetes mellitus[11–15]. There also was a randomized study comparing glimepiride, pioglitazone and metformin in Japanese patients with type 2 diabetes mellitus[16]. Given that the pathophysiology of diabetes might be different in the Japanese population compared with Western populations, further studies are needed in Japanese patients. We therefore carried out a study directly comparing the efficacy and safety of first-line therapy with glimepiride and pioglitazone in drug-naive Japanese patients with type 2 diabetes mellitus, with glycemic control as the primary end-point. We also examined other outcome measures, such as compliance with dietary/exercise therapy and dosage, bodyweight change, and, in particular, lipid changes.

MATERIALS AND METHODS

Patients

Outpatients of either sex with type 2 diabetes mellitus aged 30–75 years who were committed to a stable dietary and exercise regimen for >1 month before randomization were eligible for recruitment. HbA_{1c} had to be 6.9 to <10.4% 1 month before and at randomization, with an absolute HbA_{1c} difference <1% between these measurements. The value for HbA_{1c} (%) is estimated as a National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (JDS) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[17]. Exclusion criteria included: type 1 diabetes mellitus; use of insulin or any oral hypoglycemic agent (including an α-glucosidase inhibitor) in the month before randomization; heart failure or history of heart failure; and any serious intercurrent complication involving the heart, kidney, liver, pancreas or other organs, or hematological condition. All patients had to be sufficiently competent to give consent to participate in the study, and capable of reading, understanding and signing the informed consent form for study participation. The study was approved by the Ethics Committee of the Japan Association for Diabetes Education and Care, and was carried out in accordance with the ethical principles of the Declaration of Helsinki and its subsequent amendments.

Study Design

The present study was a multicenter (33 Japanese centers), randomized, non-blind, parallel-group trial comparing orally administered single-agent glimepiride or pioglitazone (1:1 ratio) for 6 months. Randomization was carried out by a central registration method. Patients continued their stable pre-enrollment dietary and exercise regimen throughout the study.

According to the original trial protocol, patients could receive either glimepiride or gliclazide as the sulfonylurea treatment, but in practice, all selected patients received glimepiride. The starting dose of glimepiride was 0.5 mg/day for patients with $HbA_{1c} \geq 6.9$ to <7.4%, and 1 mg/day for those with $HbA_{1c} \geq 7.4$ to <10.4%. The glimepiride dose could be increased to a maximum of 6 mg/day in order to achieve morning fasting blood glucose of <120 mg/dL. The starting dose of pioglitazone was 15 mg/day, which could be increased to a maximum of 45 and 30 mg/day in men and women, respectively, in order to achieve morning blood glucose of <120 mg/dL. The dosage of glimepiride or pioglitazone could be decreased according to the supervising physician's judgment if morning fasting blood glucose was <80 mg/dL. Drug doses were titrated according to morning fasting blood glucose measured at scheduled clinic visits.

Initiation of any anti-diabetic medication (insulin and blood glucose-lowering drugs) apart from the test drugs and any antihypertensive or antihyperlipidemic drugs was prohibited during the study period. Initiation of any other drugs was discouraged unless absolutely essential, when full details were recorded. Antihypertensive or antihyperlipidemic drugs that had been started >3 months before randomization were allowed, provided the dosage remained unchanged throughout the study; if dose change was essential, the reason and new dose were recorded.

Assessments

Full patient medical history and work-up were obtained 1 month before randomization. Patients attended morning clinical visits at baseline (month 0), at 2 weeks (month 0.5) and each month thereafter (months 1, 2, 3, 4, 5, 6). A general clinical examination of the patient was carried out at months 0, 3 and 6.

Fasting morning blood glucose was measured at each visit. HbA_{1c} was measured at month 0, 3 and 6. Fasting plasma insulin was measured at month 0 and 6. Fasting plasma lipids (total cholesterol, high-density lipoprotein [HDL]-cholesterol, low-density lipoprotein [LDL]-cholesterol and triglycerides) were measured, and bodyweight and blood pressure (BP) were recorded at months 0, 3 and 6. Fasting plasma brain natriuretic peptide (BNP) was measured at months 0 and 6. Fasting plasma insulin and plasma BNP were measured at an independent central laboratory, whereas other measurements were carried out according to routine procedures at each participating center.

Adherence to dietary and exercise therapy was categorized as 'strictly followed', 'sometimes followed' or 'not followed' at each

monthly visit. Adherence to test diabetic drug therapy was determined from returned tablet counts as 'excellent' (90–100% compliance), 'good' (70–89%), 'fair' (50–69%) and poor (<50%).

Adverse events were recorded after indirect questioning and by clinical observation. Their severity was graded as 'mild', 'moderate' or 'severe', and their potential relationship to treatment was graded as 'definite', 'probable', 'possible' or 'none'. Serious adverse events were immediately reported.

End-Points and Statistics

The primary end-point was the percentage of evaluable patients with HbA_{1c} < 6.9% at the end of the study (month 6) and a secondary end-point was the change in HbA_{1c} at 6 months compared with baseline. Other secondary end-points included changes in fasting plasma glucose, insulin, lipids and BNP, as well as bodyweight and body mass index (BMI) at month 6 compared with baseline. Safety of study medication was also a secondary end-point.

Analyses were carried out using the safety population under the headings 'study population' and 'safety' in the results, whereas analyses under the other headings in the results were carried out using the efficacy population.

Intragroup comparison of data over time vs baseline was analyzed by paired *t*-test, whereas intergroup comparisons were analyzed by one-way analysis of variance (ANOVA) or χ^2-test. A two-tailed level of significance was accepted for *P*-values < 0.05. Data are generally presented as mean values standard deviation (SD) or categorical values.

The percentages of patients rated as strictly adherent or sometimes adherent to dietary/exercise therapy and those who had excellent or good adherence to the study anti-diabetic drug therapy were calculated for each month. The median (range) percentages over the 6-month study treatment period were then calculated.

RESULTS

Study Population

The study was carried out from 1 August 2007 to 28 February 2010. A total of 238 patients were initially screened, of whom 191 were eventually randomized to treatment. Figure 1 shows the disposition of the patients during screening, at randomization, and in the safety and efficacy populations, as well as the reasons for dropout. The safety population included all patients initially randomized to glimepiride (n = 95) and pioglitazone (n = 96), whereas the efficacy population included 86 patients in the glimepiride group and 91 patients in the pioglitazone group. The baseline demographic and clinical characteristics of the patients in the two groups are shown in Table 1. There were no statistically significant differences in baseline characteristics between the groups, except for mean HDL-cholesterol, which was significantly higher (59.3 ± 23.0 vs 52.8 ± 13.7 mg/dL, P = 0.024), and triglycerides, which was significantly lower (129.8 ± 68.4 vs 164.0 ± 112.4 mg/dL, P = 0.014), in the group receiving glimepiride.

Glucose Markers

Similar percentages of patients in the glimepiride and pioglitazone groups attained the primary end-point (HbA_{1c} < 6.9% at month 6): 61.2% (52/85) vs 56.8% (50/88), respectively (P = 0.64; Figure 2a). Mean HbA_{1c} was significantly (P < 0.001) decreased at months 3 and 6 vs baseline in both the glimepiride and pioglitazone groups (Figure 2b). Mean HbA_{1c} was significantly lower in the glimepiride group as compared with those receiving pioglitazone (6.9 ± 0.7% vs 7.3 ± 1.0%; P = 0.022) at month 3, but not at month 6 (Figure 2b). There was no

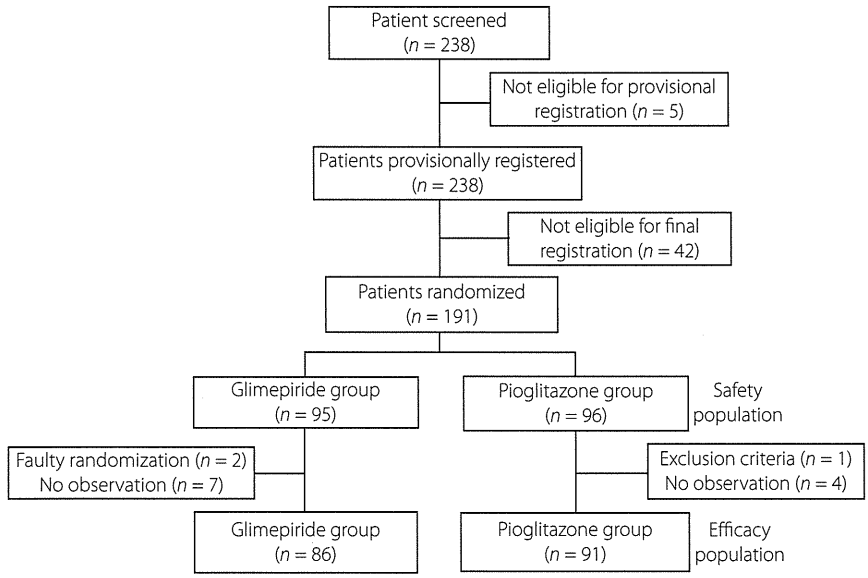

Figure 1 | Patient disposition.

Table 1 | Baseline demographic and clinical characteristics of the study patients

Characteristic	Glimepiride (n = 95)	Pioglitazone (n = 96)
Sex (male/female)	62/33	65/31
Age (years)	57.7 ± 10.4 (n = 95)	56.8 ± 10.3 (n = 96)
Body weight (kg)	65.6 ± 12.5 (n = 93)	65.5 ± 14.6 (n = 92)
Body mass index (kg/m^2)	24.6 ± 3.8 (n = 93)	24.5 ± 4.3 (n = 92)
Duration of diabetes (years)	6.0 ± 8.2 (n = 41)	4.1 ± 4.3 (n = 52)
Fasting glucose (mg/dL)	143.1 ± 39.8 (n = 90)	145.8 ± 45.6 (n = 90)
HbA_{1c} (%)	7.8 ± 0.9 (n = 95)	7.8 ± 0.9 (n = 95)
Fasting insulin (μU/mL)	8.3 ± 9.4 (n = 95)	8.6 ± 12.2 (n = 94)
HOMA-β	41.7 ± 71.2 (n = 90)	56.9 ± 108.0 (n = 89)
HOMA-R	3.0 ± 4.3 (n = 90)	2.5 ± 2.6 (n = 89)
Total cholesterol (mg/dL)	207.5 ± 39.1 (n = 81)	205.5 ± 38.2 (n = 87)
LDL-cholesterol (mg/dL)	126.5 ± 36.5 (n = 79)	123.2 ± 32.6 (n = 79)
HDL-cholesterol (mg/dL)	59.3 ± 23.0 (n = 90)*	52.8 ± 13.7 (n = 88)*
Triglycerides (mg/dL)	129.8 ± 68.4 (n = 91)†	164.0 ± 112.4 (n = 91)†
Brain natriuretic peptide (pg/mL)	27.8 ± 88.2 (n = 94)	20.6 ± 39.6 (n = 94)

Data are number of patients (categorized data) or mean ± SD (qualitative data). Qualitative data are missing for some patients in treatment groups: numbers with available data are shown in parentheses. These baseline observational data may differ from data used for paired t-test analyses.
*High-density lipoprotein (HDL)-cholesterol was significantly different between the groups (P = 0.024).
†Triglycerides were significantly different between the groups (P = 0.014). HbA_{1c}, glycated hemoglobin; HDL, high-density lipoprotein; HOMA-β, homeostasis model assessment for β-cell function; HOMA-R, homeostasis model assessment for insulin resistance; LDL, low-density lipoprotein.

significant (P = 0.31) difference between the groups in the decrease in mean HbA_{1c} at month 6 vs baseline (Figure 2c).

There were no statistically significant differences between the groups in changes in fasting plasma glucose, fasting insulin, homeostasis model assessment for β-cell function (HOMA-β) and homeostasis model assessment for insulin resistance (HOMA-R) (Figure 3) at month 6 vs baseline. There was a tendency for fasting blood glucose to improve more in the glimepiride group as compared with the pioglitazone group at month 6 vs baseline (−21.2 ± 33.7 vs −12.5 ± 47.7 mg/dL; P = 0.17).

Plasma Lipids

Changes in mean plasma lipid concentrations (total cholesterol, HDL-cholesterol, LDL-cholesterol and trigylcerides) at month 6 from baseline are shown in Figure 4 for the treatment groups, which showed significant differences in total cholesterol

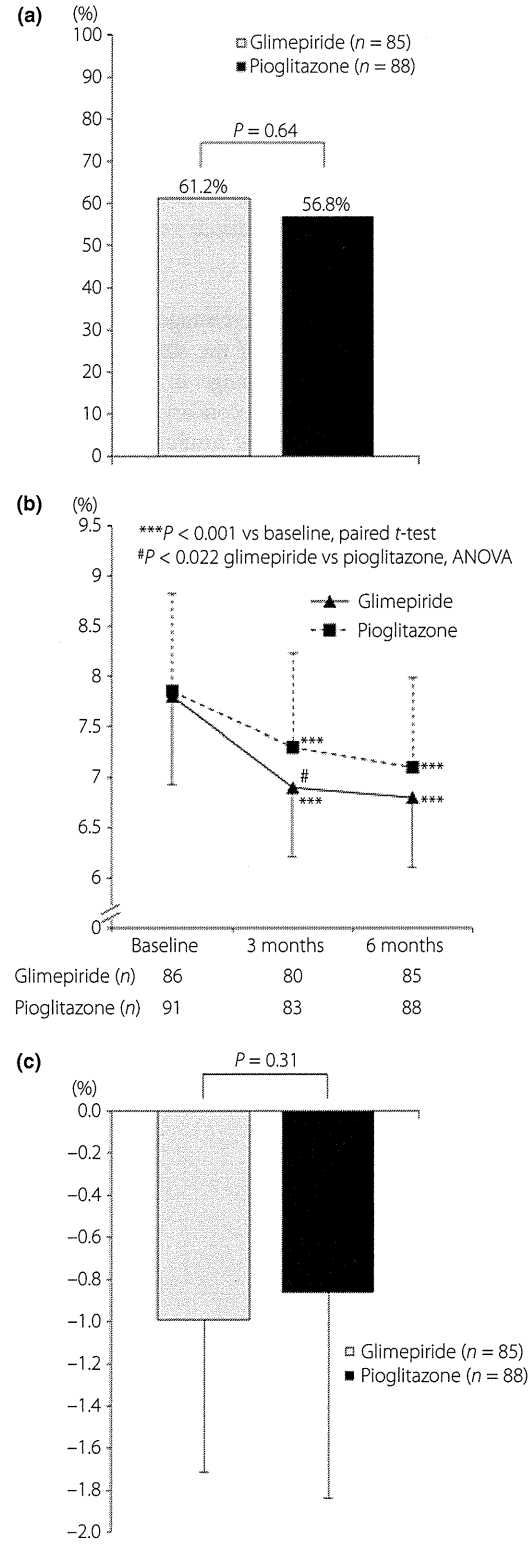

Figure 2 | Glycated hemoglobin (HbA_{1c}). (a) Percentage of patients with HbA_{1c} < 6.9% at 6 months. (b) Change in mean (±SD) HbA_{1c} during the study. (c) Change in mean (±SD) HbA_{1c} at month 6 vs baseline.

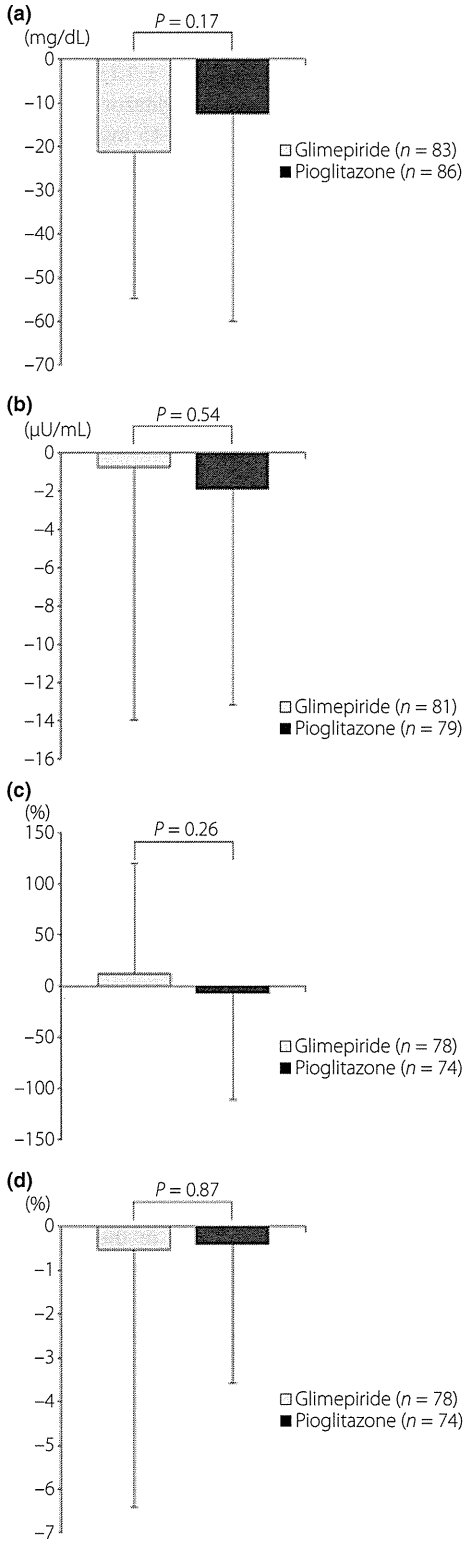

Figure 3 | Change in mean (±SD) fasting (a) glucose, (b) insulin, (c) homeostasis model assessment for β-cell function and (d) homeostasis model assessment for insulin resistance at month 6 vs baseline.

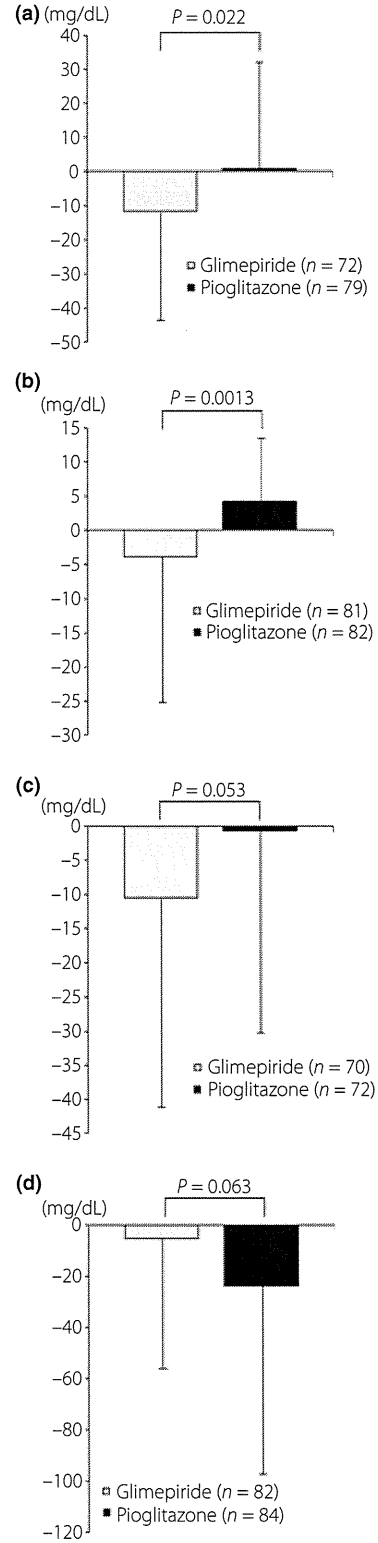

Figure 4 | Change in mean (±SD) fasting (a) total cholesterol, (b) high-density lipoprotein cholesterol, (c) low-density lipoprotein cholesterol and (d) triglycerides at month 6 vs baseline.

(−11.6 ± 32.4 vs 0.8 ± 32.9 mg/dL, $P = 0.022$) and HDL-cholesterol (−3.9 ± 20.7 vs 4.2 ± 8.7 mg/dL, $P = 0.0013$) between the glimepiride and pioglitazone groups, respectively. The changes for LDL-cholesterol (−10.5 ± 30.4 vs −0.7 ± 29.7 mg/dL, $P = 0.053$) and triglycerides (−5.3 ± 49.8 vs −23.8 ± 74.4 mg/dL, $P = 0.063$) approached significance comparing glimepiride and pioglitazone, respectively. At month 6, the following significant ($P < 0.05$) intragroup changes in mean plasma lipid concentrations were noted as compared with baseline: total cholesterol decreased from 203.5 ± 37.4 to 195.5 ± 36.0 mg/dL ($P = 0.036$) and LDL-cholesterol decreased from 124.5 ± 32.8 to 116.3 ± 32.7 mg/dL ($P = 0.028$) in the glimepiride group, whereas HDL-cholesterol increased from 51.6 ± 13.1 to 56.0 ± 13.7 mg/dL ($P < 0.0001$) and triglyceride decreased from 167.6 ± 120.2 to 143.6 ± 93.8 mg/dL ($P = 0.0079$) in the pioglitazone group.

Bodyweight and Body Mass Index

There were no statistically significant intergroup differences for changes in mean bodyweight or BMI at month 6 vs baseline. At month 6, there were significant intragroup increases in bodyweight (from 65.5 ± 15.1 to 66.2 ± 14.4 kg, $P = 0.036$) and BMI (from 24.5 ± 4.5 to 24.9 ± 4.3 kg/m^2, $P = 0.016$) compared with baseline for patients in the pioglitazone group. The intragroup changes from baseline to month 6 in the glimepiride group were not statistically significant for bodyweight (from 66.0 ± 12.0 to 66.4 ± 11.7 kg) and BMI (from 24.6 ± 3.6 to 24.8 ± 3.6 kg/m^2).

Brain Natriuretic Peptide

At month 6, the change in mean plasma BNP from baseline (−2.6 ± 37.5 vs 6.0 ± 13.9 mg/dL, $P = 0.060$) approached significance comparing glimepiride and pioglitazone, respectively. There was a significant intragroup increase in mean BNP (from 17.3 ± 14.6 to 23.3 ± 22.1 pg/mL, $P = 0.0003$) at month 6 compared with baseline in the pioglitazone group, whereas the change in the glimepiride group was not statistically significant (from 28.6 ± 93.9 to 26.0 ± 62.7 pg/mL).

Adherence to Dietary, Exercise and Anti-diabetic Therapy

Good adherence (strictly adherent or sometimes adherent) to dietary therapy was shown by a median of 89.5% (range 88.2–93.0%) and 91.7% (range 89.3–93.3%) of patients in the glimepiride and pioglitazone groups, respectively. Good adherence to exercise therapy was shown by a median of 86.0% (range 84.9–88.4%) and 83.9% (range 82.2–86.5%) of patients in the glimepiride and pioglitazone groups, respectively. Good adherence (excellent or good) to anti-diabetic medication was shown by a median of 95.9% (range 93.6–97.7%) and 96.5% (range 96.3–98.9%) of patients in the glimepiride and pioglitazone groups, respectively.

Dosage

At month 6, the mean daily drug dosage was 1.51 ± 1.27 mg (range 0.25–6 mg) in the glimepiride group and 23.24 ± 11.40 mg (range 7.5 to −45 mg) in the pioglitazone group. The mean daily glimepiride dosage at month 6 was 1.59 ± 1.33 mg (range 0.25–6 mg) in men and 1.32 ± 1.13 mg (range 0.25–4 mg) in women. The mean daily pioglitazone dosage at month 6 was 25.04 ± 12.52 (range 7.5–45 mg) in men and 19.40 ± 7.37 (range 7.5–30 mg) in women.

Safety

There were no severe or serious adverse events in either group. Seven patients in the glimepiride group and five patients in the pioglitazone group experienced blood glucose concentrations <60 mg/dL, with no statistically significant difference between the groups. The only other adverse events possibly related to treatment were mild or moderate in intensity: four patients with edema in the pioglitazone group and constipation in one patient in the glimepiride group.

DISCUSSION

The present study showed that there was no clear difference between first-line, single-agent glimepiride and pioglitazone therapy in Japanese patients with type 2 diabetes mellitus with respect to glycemic control as determined from the primary end-point, the rate of attaining target $HbA_{1c} < 6.9\%$: 61.2 vs 56.8%, respectively ($P = 0.64$). However, there was an indication that the onset of action of glimepiride might be faster than that of pioglitazone, because the mean HbA_{1c} was significantly lower in patients receiving glimepiride as compared with those receiving pioglitazone (6.9 ± 0.7% vs 7.3 ± 1.0%; $P = 0.022$) at month 3, whereas it was comparable in both groups (~6.9%) at month 6. Previous comparison of glimepiride and pioglitazone in Japanese patients with type 2 diabetes mellitus showed similar results[16]. These patients had higher baseline mean HbA_{1c} (~10%; JDS) and end-point HbA_{1c} at month 12 (~7.8%; JDS) than the patients in the present study, although the reduction in mean HbA_{1c} was similar in both treatment groups at end-point, as in the present study. The authors also noted a slower decrease in HbA_{1c} with pioglitazone, with maximal reduction requiring about 6 months. The slower onset of glycemic control with pioglitazone compared with glimepiride has been reported elsewhere[11,13]. We found no statistically significant differences between pioglitazone and glimepiride in the present study for changes in fasting blood glucose, fasting insulin, HOMA-β and HOMA-R at month 6. Other comparative studies of pioglitazone and glimepiride have shown significant reductions in fasting blood glucose and insulin, associated with significant decreases in insulin resistance, with pioglitazone, but not with glimepiride[11,12]. Possible reasons for these inconsistent results might be related to the study duration or the patient background. The study duration in the trial by Tan *et al.*[11] was 52 weeks, which is longer than the present study. The trial by Langenfeld *et al.*[12], which primarily assessed the decrease of carotid intima-media thickness, enrolled more obese subjects (mean BMI 31.8 kg/m^2) than the present trial (mean BMI 24.6 kg/m^2). Further studies that set the reduction of insulin

resistance as the primary end-point are warranted to resolve this possible inconsistency.

With respect to plasma lipid profile, the present study showed that glimepiride significantly decreased total cholesterol and LDL-cholesterol, which is consistent with other studies of Asian[18,19] or Western[12,13] patients. Reports on the effect of glimepiride on HDL-cholesterol in Japanese or Chinese patients have been less consistent, showing either no effect[4,16] or a significant increase[18]. In the present study, glimepiride showed a trend towards decreasing HDL-cholesterol, but this did not reach statistical significance. However, the changes of HDL-cholesterol levels in the glimepiride group were within normal ranges, which suggest that these changes might not be clinically relevant. A possible reason for HDL-cholesterol not increasing in the glimepiride group might be that baseline HDL-cholesterol levels were higher than those of the pioglitazone group. Pioglitazone showed a significant increase in HDL-cholesterol in the present study, which has been the only consistent effect on plasma lipids shown in other studies of pioglitazone in Japanese[20] or Western[12,14,15] patients. Glimepiride has been shown to exert a prophylactic effect on atherosclerosis in cholesterol-fed rabbits[21]. Glimepiride has also been shown to normalize the adverse serum and hepatic lipid profile induced by a simulated Western-like diet in rats, possibly as a result of decreasing very low-density lipoprotein synthesis and increasing LDL catabolism through insulin secretion[22]. Research to elucidate the molecular biological mechanism involved is awaited.

The adherence to diet and exercise regimens was good in the present study, which probably contributed to some degree to the good maintenance of glycemic control and bodyweight. In fact, bodyweight and BMI did not change significantly in the group receiving glimepiride, whereas some concern was raised about possible bodyweight gain during glimepiride therapy in a previous Japanese study[4]. A German study in a general practice setting has shown a BMI-dependent reduction in bodyweight in patients on glimepiride therapy[23]. There was, however, a statistically significant, but relatively minor, increase in bodyweight and BMI in the group receiving pioglitazone. One of the possible reasons for the lack of marked weight gain in each group is that the patients adhered well to the dietary and exercise therapy prescribed by the doctors.

Both glimepiride and pioglitazone were well tolerated in the present study. No severe or serious adverse events were reported. The only symptomatic adverse events possibly related to treatment were mild or moderate in intensity and were limited to edema in the pioglitazone group (n = 4) and constipation in the glimepiride group (n = 1). Similar numbers of patients experienced asymptomatic hypoglycemia (<60 mg/dL) in the glimepiride and pioglitazone groups (n = 7 and 5, respectively).

There were several limitations to the present study, including the relatively small sample size, which limited the power to detect potential differences between the study treatments. Furthermore, it was essentially an open-label (non-blind) design with the drugs being administered as commercially available drugs. This might have led to an overestimation of adverse events, as both the patients and investigators could have been aware of the known adverse events of the study medications. In addition to adverse events, effectiveness might have been biased as a result of non-blindness, although the primary end-point measurement, HbA_{1c}, is an objective assessment of efficacy unlikely to be affected by subjective bias. In addition, the study background was essentially a real-life situation, in which patients were treated according to normal clinical practice. However, it is possible that dose titration of the study medications might have been insufficient. In fact, the mean daily dose of glimepiride (1.5 mg) at month 6 would appear to be somewhat lower than we would have expected. It is possible that the present study included a higher proportion of patients with milder disease, although a more probable reason for the relatively lower dose might be a result of the background of our patients, who attained comparatively good blood glucose control which, in turn, was related to their excellent adherence to dietary and exercise therapy.

In conclusion, we found that there was no statistically significant difference between glimepiride and pioglitazone with respect to glycemic control as shown by the rate of attaining target HbA_{1c} < 6.9%. Both of these anti-diabetic agents were similarly well tolerated. Weight gain and increased insulin resistance, which have been considered a concern with sulfonylurea therapy, were not observed in the present study. Glimepiride significantly lowered total cholesterol and LDL-cholesterol, whereas pioglitazone increased HDL-cholesterol, which is consistent with previous reports. Based on these findings, it is recommended that when selecting first-line, single-agent drug therapy for patients with type 2 diabetes mellitus, the individual status of the patients, including their adherence to dietary/exercise therapy, dose required, bodyweight, serum lipid profile and general condition, is considered.

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REFERENCES

1. National Institute of Health and Nutrition. Outline for the results of the National Health and Nutrition Survey Japan, 2007. Available at: http://www.nih.go.jp/eiken/english/research/pdf/nhns2007.pdf [accessed on 2 August 2010].
2. Kanatsuka A, Kawai K, Hirao K, *et al.* Research on antihyperglycemic therapies in patients with type 2 diabetes mellitus in Japan (I): drug therapies and actual drug use. *J Jpn Diabetes Soc* 2006; 49: 409–415 (Japanese).
3. Holman RR, Paul SK, Bethel MA, *et al.* 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med* 2008; 359: 1577–1589.
4. Toyota T, Satoh J, Fukao A, *et al.* Clinical evaluation of glimepiride (HOE490) in non-insulin-dependent diabetes mellitus – a double-blind placebo-controlled study/phase III additional study. *J Clin Ther Med* 1997; 13: 157–158 (Japanese).
5. Draeger KE, Wernicke-Panten K, Lomp H-J, *et al.* Long-term treatment of type 2 diabetic patients with the new oral antidiabetic agent glimepiride (Amaryl): a double-blind comparison with glibenclamide. *Horm Metab Res* 1996; 28: 419–425.
6. Dormandy JA, Charbonnel B, Eckland DJA, *et al*; on behalf of the PROactive investigators. Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. *Lancet* 2005; 366: 1279–1289.
7. Nissen SE, Wolski K. Effect of rosiglitazone on the risk of myocardial infarction and death from cardiovascular causes. *N Engl J Med* 2007; 356: 2457–2471.
8. Lincoff AM, Wolski K, Nicholls SJ, *et al.* Pioglitazone and risk of cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis of randomized trials. *JAMA* 2007; 298: 1180–1188.
9. Kawamori R, Kadowaki T, Onji M, *et al.* Hepatic safety profile and glycaemia control of pioglitazone in more than 20,000 patients with type 2 diabetes mellitus: postmarketing surveillance study in Japan. *Diabetes Res Clin Pract* 2007; 76: 229–235.
10. Loke YK, Singh S, Furnberg CD. Long-term use of thiazolidinediones and fractures in type 2 diabetes: a meta-analysis. *CMAJ* 2009; 180: 32–39.
11. Tan M, Johns D, González Gálvez G, *et al*; on behalf of the GLAD Study Group. Effects of pioglitazone and glimepiride on glycemic control and insulin sensitivity in Mexican patients with type 2 diabetes mellitus: a multicenter, randomized, double-blind, parallel-group trial. *Clin Ther* 2004; 26: 680–693.
12. Langenfeld MR, Forst T, Hohberg C, *et al.* Pioglitazone decreases carotid intima-media thickness independently of glycemic control in patients with type 2 diabetes mellitus: results from a controlled randomized study. *Circulation* 2005; 111: 2525–2531.
13. Umpierrez G, Issa M, Vlajnic A. Glimepiride versus pioglitazone combination therapy in subjects with type 2 diabetes inadequately controlled on metformin monotherapy: results of a randomized clinical trial. *Curr Med Res Opin* 2006; 22: 751–759.
14. Mazzone T, Meyer PM, Feinstein SB, *et al.* Effect of pioglitazone compared with glimepiride on carotid intima-media thickness in type 2 diabetes: a randomized trial. *JAMA* 2006; 296: 2572–2581.
15. Nissen SE, Nichols SJ, Wolski K, *et al*; for the PERISCOPE Investigators. Comparison of pioglitazone vs glimepiride on progression of coronary atherosclerosis in patients with type 2 diabetes: the PERISCOPE randomized controlled trial. *JAMA* 2008; 299: 1561–1573.
16. Yamanouchi T, Sakai T, Igarashi K, *et al.* Comparison of metabolic effects of pioglitazone, metformin, and glimepiride over 1 year in Japanese patients with newly diagnosed Type 2 diabetes. *Diabet Med* 2005; 22: 980–985.
17. Seino Y, Nanjo K, Tajima N, *et al.* Report of the committee on the classification and diagnostic criteria of diabetes mellitus. *J Diabetes Invest* 2010; 1: 212–228.
18. Araki T, Emoto M, Konishi T, *et al.* Glimepiride increases high-density lipoprotein cholesterol via increasing adiponectin levels in type 2 diabetes mellitus. *Metabolism* 2009; 58: 143–148.
19. Xu D-Y, Zhao S-P, Huang Q-X, *et al.* Effects of glimepiride on metabolic parameters and cardiovascular risk factors in patients with newly diagnosed type 2 diabetes mellitus. *Diabetes Res Clin Pract* 2010; 88: 71–75.
20. Kaneko T, Baba S, Toyota T. Clinical evaluation of an insulin-resistance improving agent, AD-4833, in patients with non-insulin dependent diabetes mellitus (NIDDM) on diet therapy alone. A placebo controlled double blind clinical study. *Jpn J Clin Exp Med* 1997; 74: 1515–1539 (Japanese).
21. Shakuto S, Oshima K, Tsuchiya E. Glimepiride exhibits prophylactic effect on atherosclerosis in cholesterol-fed rabbits. *Atherosclerosis* 2005; 182: 209–217.
22. Schaalan M, El-Abhar HS, Barakat M, *et al.* Westernized-like-diet-fed rats: effect on glucose homeostasis, lipid profile, and adipocyte hormones and their modulation by rosiglitazone and glimepiride. *J Diabetes Complications* 2009; 23: 199–208.
23. Scholz GH, Schneider K, Knirsch W, *et al.* Efficacy and tolerability of glimepiride in daily practice: a non-interventional observational cohort study. *Clin Drug Invest* 2001; 21: 597–604.

Impact of endogenous and exogenous insulin on basal energy expenditure in patients with type 2 diabetes under standard treatment[1–3]

Kaori Ikeda, Shimpei Fujimoto, Masashi Goto, Chizumi Yamada, Akihiro Hamasaki, Kenichiro Shide, Takashi Kawamura, and Nobuya Inagaki

ABSTRACT

Background: Factors that affect resting energy expenditure or basal energy expenditure (BEE) in patients with type 2 diabetes under standard treatment have not been evaluated in detail.

Objective: We determined the clinical factors that affected BEE in addition to body composition in patients with type 2 diabetes under standard treatment.

Design: BEE was measured by using indirect calorimetry under a strict basal condition in 58 Japanese patients with type 2 diabetes after >7 d as inpatients under management of diabetes with medical nutrition therapy and medications. Insulin secretion was measured with a glucagon test. Stepwise regression was applied to explore determinants of BEE.

Results: In the stepwise estimation, insulin secretion ($P = 0.015$), insulin therapy ($P = 0.012$), and pulse rate ($P = 0.011$) were selected in addition to fat-free mass (FFM) ($P < 0.001$) and fat mass ($P = 0.006$) as significant independent determinants of BEE. Standardized partial regression coefficients of the additional 3 factors were −0.16, −0.15, and 0.15, respectively, whereas those for FFM and fat mass were 0.82 and 0.19, respectively. The additional 3 factors explained another 3.9% of the variability of BEE, and the adjusted coefficient of determination was 83.4%. Age, sex, other medications, and parameters of glycemic control were not significant determinants beyond the combined contribution of body composition, endogenous and exogenous insulin, and pulse rate.

Conclusion: Endogenous insulin secretion and exogenous insulin administered in treatment have significant independent effects in the lowering of BEE in patients with diabetes under standard management with medical nutrition therapy and medications. *Am J Clin Nutr* 2011;94:1513–8.

INTRODUCTION

MNT[4] is the basis and starting point of treatment of all patients with diabetes, and the failure of MNT alone may predict the inability to attain optimal glycemic control. In addition, in obese patients with diabetes, MNT aims at weight control to improve insulin resistance and avoid obesity-related health problems. Therefore, estimation of the daily energy expenditure of patients is necessary for individualized diabetic meal plans. For example, MNT for obese patients provides a diet of 500–1000 kcal less energy than the estimated energy expenditure (1). REE or BEE is defined as the minimal amount of energy expended to maintain metabolic activities of cells, tissues, and organs and represents a large component of daily energy expenditure. REE or BEE is used to estimate total energy expenditure as a multiple of REE or BEE according to daily activity.

In healthy subjects, 65–90% of the interindividual variation in REE is explained by FFM or high-metabolic-rate organ mass (2, 3). In patients with diabetes, FFM is also assumed to be a main factor that affects REE and BEE. The influence of the diabetic condition on energy expenditure has also been examined. After adjustment for FFM, REE and BEE in patients with diabetes were shown to be greater than those in healthy control (4, 5). In contrast, no difference was reported in FFM-adjusted REE between mildly hyperglycemic patients and control subjects (6). In addition, a treatment-induced reduction of REE has been reported in type 1 and type 2 diabetes (7, 8). However, the factors that affect REE or BEE in patients with diabetes under standard treatment, which are the more important in clinical practice, have not been precisely evaluated.

In most studies that evaluated energy expenditure, REE has been measured rather than BEE. However, resting conditions are defined less rigorously than are basal conditions, and REE can include components that involve physical or psychological stress and variations in ambient and body temperature (9–11). BEE is measured early in the morning before the subject has engaged in any physical activity and ≥10 h after ingestion of any food, drink, or nicotine and remains remarkably constant on a daily basis (9, 11). We applied the measurement of BEE under such

[1] From the Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan (KI, SF, CY, AH, KS, and NI), and Kyoto University Health Service, Kyoto, Japan (MG and TK).

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[3] Address reprint requests and correspondence to S Fujimoto, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: fujimoto@metab.kuhp.kyoto-u.ac.jp.

[4] Abbreviations used: A_{1c}, glycohemoglobin; BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection; EGO, endogenous glucose output; FFM, fat-free mass; FPG, fasting plasma glucose; Hb A_{1c}, glycated hemoglobin; MNT, medical nutrition therapy; PG, plasma glucose; PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE; REE, resting energy expenditure.

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strict basal conditions to minimize the interindividual variation derived from methodologic or external unknown factors.

In the current study, clinical factors that might affect BEE in patients with diabetes under standard treatment were evaluated.

SUBJECTS AND METHODS

Subjects

Japanese patients with type 2 diabetes who were admitted to the Department of Diabetes and Clinical Nutrition, Kyoto University Hospital, Kyoto, Japan, for diabetes self-management education during the period of December 2007 through September 2009 were recruited. The study protocol was approved by the ethics committee of Kyoto University. Written informed consent was obtained from all participants.

During admission, which averaged ~18 d, participants took MNT with or without medications, including oral hypoglycemic agents and insulin, according to the treatment guide for diabetes of the Japan Diabetes Society (12). The physical activity of subjects was not restricted, but participants did not engage in intensive exercise. Participants were screened by a medical history, physical examination, and laboratory examination to ensure the absence of hepatic, renal (including macroalbuminurea), pulmonary, thyroid, and cardiac dysfunction, inflammation, and malignant tumors. Participants who took steroids or β-blockers and subjects who had physical disabilities were excluded.

Energy expenditure

BEE was measured in the morning >7 d after admission under glycemic control with MNT (29.0 ± 2.6 kcal/kg of standard body weight/d that consisted of 52% carbohydrate, 20% protein, and 28% fat in energy-intake percentages) and with without prescribed medications. Standard body weight (in kg) was calculated by multiplying 22 (kg/m^2) by square of height (in m). Premenopausal women were studied during their follicular phase within 7 d after the last day of menstruation.

Whole-body oxygen consumption and carbon dioxide production were measured for >10 min with indirect calorimetry (AE300S; Minato Medical Science) by one investigator at the bedside of each participant under the strict condition of the methods described previously (9, 10, 13). Briefly, afebrile patients in a postabsorptive state after an overnight fast (14 h) with <10 mmol capillary PG/L before the measurement remained in a supine position after waking on the bed in the ward without smoking or taking caffeine, and measurements were performed at room temperature between 22°C and 27°C. After discarding the initial 5 min of data, we adopted 5 consecutive minutes of data from the rest in accord with the steady state definition (10) during which the CV for oxygen consumption and carbon dioxide production was achieved at ≤10% and applied them to the Weir formula together with 24-h urinary urea nitrogen (14).

Body composition

Height was measured on the day of admission, and body weight was measured immediately after measurement of BEE. FFM and fat mass were measured with a dual energy X-ray absorptiometry scanner (Discovery; Hologic) within 3 d before or after the measurement of BEE.

Clinical and metabolic factors

The duration of diabetes was determined from medical records, medical histories, and previous clinical data according to the criteria for the diagnosis of diabetes proposed by the American Diabetes Association (15). Hb A_{1c} was measured by using HPLC (ADAMS A1C HA8180; Arcray) and expressed as a National Glycohemoglobin Standardization Program equivalent value (A_{1c}; percentage) calculated by the formula

$$\text{Percentage of } A_{1c} = \text{percentage of Hb } A_{1c} \text{ (Japan Diabetes Society)} + 0.4\% \quad (1)$$

which considers the relational expression of Hb A_{1c} (Japan Diabetes Society) (percentage) measured by using the previous Japanese standard substance and measurement methods and Hb A_{1c} (National Glycohemoglobin Standardization Program) (16). Thyroid stimulating hormone was measured by using electrochemiluminescence immunoassay (Elecsys Anti-Tg; Roche Diagnostics GmbH) to confirm the absence of thyroid dysfunction. Capillary glucose before each meal was measured with a glucose meter (One Touch Ultra; Johnson & Johnson) and expressed as capillary PG. As a variable of glycemic control, the mean preprandial PG for 3 consecutive days before the measurement of BEE and FPG just before the measurement of BEE was used. The mean pulse rate was calculated from the records of checkups by nurses on 3 consecutive days including the day of BEE measurement.

Insulin secretion

β cell function was evaluated after an overnight fast by measuring CPR6′ (17) because the test is valid in patients who take insulin therapy. The serum C-peptide immunoreactivity was measured by using an immunoenzymometric assay (ST AIA-PACK C-peptide; Toso). On the morning of the glucagon test, participants took their medication after the test.

Statistical analysis

Descriptive data were expressed as means ± SDs. Information of medications, including insulin, sulfonylurea, and metformin, was coded as use = 1 and nonuse = 0. Sex was coded as male = 1 and female = 0. The Mann-Whitney U and Fisher's exact tests were performed to identify differences in the characteristics of men and women. The interrelation between BEE and clinical factors was investigated by means of Kendall's rank correlation coefficients. Clinical factors include body composition (FFM and fat mass), FPG, mean preprandial PG, A_{1c}, insulin secretion, mean pulse rate, dietary energy, and medications (ie, insulin, sulfonylurea, and metformin). A multiple linear regression analysis was performed to evaluate the contribution of each determinant to BEE. Variables were selected by stepwise estimation from age, sex, and the clinical factors previously described. When P was <0.05 and ≥0.15, the variable was added and removed, respectively. Data were analyzed by use of Stata 11.0 software (StataCorp). Statistical significance was set at $P < 0.05$ (2-tailed).

TABLE 1
Characteristics of participants[1]

	All	Men	Women
Patients (*n*)	58	35	23
Age (y)[2]	60.2 ± 9.2[3]	57.6 ± 9.7	64.0 ± 7.1
Body weight (kg)[2]	63.7 ± 15.6	68.3 ± 16.5	56.8 ± 11.2
BMI (kg/m^2)	24.2 ± 5.0	24.1 ± 5.6	24.4 ± 4.1
FFM (kg)[2]	48.4 ± 11.1	54.1 ± 9.4	39.7 ± 7.1
BEE (kcal/d)[2]	1294 ± 227	1397 ± 217	1136 ± 135
CPR6′ (ng/mL)	3.4 ± 1.8	3.4 ± 2.0	3.3 ± 1.6
FPG (mg/dL)	114.5 ± 25.2	113.7 ± 25.1	115.6 ± 25.9
PPPG (mg/dL)	143.9 ± 35.5	147.5 ± 37.6	138.5 ± 32.0
A_{1c} (%)	10.5 ± 2.5	10.3 ± 2.4	10.9 ± 2.7
Pulse rate (beats/min)	72.7 ± 10.1	75.0 ± 10.8	69.2 ± 7.8
Duration of diabetes (y)	8.4 ± 7.0	10.0 ± 7.9	5.9 ± 4.4
Treatment			
Diet (kcal · SBW^{-1} · d^{-1})	28.8 ± 2.4	28.7 ± 2.0	29.0 ± 3.0
Medications (*n*)			
Insulin only	24	17	7
Insulin + metformin	10	4	6
Insulin + sulfonylurea	3	2	1
Insulin + sulfonylurea + metformin	1	0	1
Sulfonylurea only	8	5	3
Sulfonylurea + metformin	5	2	3
Metformin only	4	3	1
None	3	2	1

[1] A_{1c}, glycohemoglobin; BEE, basal energy expenditure; CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection; FFM, fat-free mass; FPG, fasting plasma glucose just before the measurement of BEE; PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE; SBW, standard body weight.
[2] Mann-Whitney *U* test revealed significant ($P < 0.05$) differences between men and women.
[3] Mean ± SD (all such data).

RESULTS

Participant characteristics and results of measurement are shown in **Table 1**. BMI (in kg/m^2), CPR6′, FPG, PPPG, A_{1c}, pulse rate, duration of diabetes, and treatment did not differ significantly between men and women, whereas body weight, FFM, and BEE were significantly higher in men, and age was higher in women.

Significant positive correlations were observed between BEE and FFM ($r = 0.72$) and dietary energy ($r = 0.33$), but dietary energy also had a significant positive correlation with FFM ($r = 0.37$) and a significant negative correlation with fat mass ($r = -0.38$) (**Table 2**). Fat mass had a significant negative correlation with mean preprandial PG ($r = -0.30$) and a significant positive correlation with insulin secretion (CPR6′) ($r = 0.43$)

TABLE 2
Correlations between BEE and possible determinants of BEE[1]

	BEE	FFM	FM	FPG	PPPG	A_{1c}	CPR6′	PR	Diet	Insulin[2]	Sulfonylurea[2]
BEE	1.00	—	—	—	—	—	—	—	—	—	—
FFM	0.72*	1.00	—	—	—	—	—	—	—	—	—
FM	0.18†	0.16	1.00	—	—	—	—	—	—	—	—
FPG	−0.05	−0.08	−0.13	1.00	—	—	—	—	—	—	—
PPPG	−0.08	−0.08	−0.30*	0.42*	1.00	—	—	—	—	—	—
A_{1c}	−0.07	−0.03	−0.14	0.16	0.18†	1.00	—	—	—	—	—
CPR6′	0.19†	0.19†	0.43*	0.03	−0.23†	−0.09	1.00	—	—	—	—
PR	0.21†	0.14	0.05	0.07	0.08	−0.06	0.07	1.00	—	—	—
Diet	0.33*	0.37*	−0.38*	0.07	0.20†	0.06	−0.14	0.09	1.00	—	—
Insulin[2]	−0.12	−0.06	−0.18	0.28†	0.37*	0.27†	−0.24†	0.13	0.00	1.00	—
Sulfonylurea[2]	0.01	−0.00	0.12	−0.36‡	−0.28†	−0.11	0.07	−0.15	−0.06	−0.57*	1.00
Metformin[2]	0.18	0.15	0.40*	−0.13	−0.38*	−0.01	0.29‡	0.05	−0.22	−0.16	0.01

[1] A_{1c}, glycohemoglobin; BEE, basal energy expenditure (kcal/d); CPR6′, C-peptide immunoreactivity 6 min after intravenous glucagon injection (ng/mL); Diet, energy of diet (kcal/d); FFM, fat-free mass (kg); FM, fat mass (kg); FPG, fasting plasma glucose just before the measurement of BEE (mg/dL); PPPG, mean preprandial plasma glucose for 3 consecutive days before the measurement of BEE (mg/dL); PR, pulse rate (beats/min). *†,‡Kendall's correlation coefficients ($n = 58$): *$P < 0.001$, †$P < 0.05$, ‡$P < 0.01$.
[2] Use = 1; nonuse = 0.