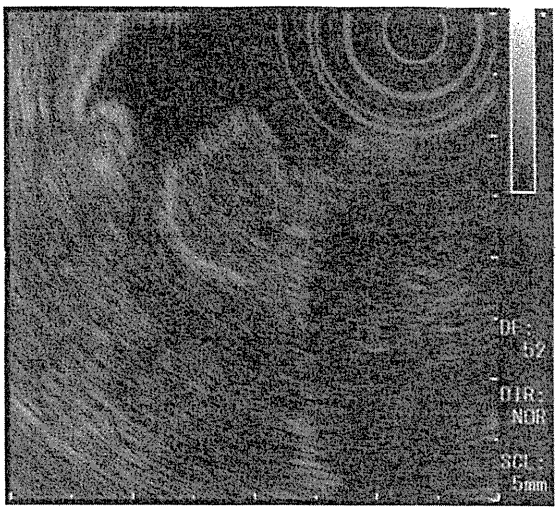

Figure 7 症例2の超音波内視鏡検査所見（20Mhz）.
病巣の主座は第3層にあり，多房性無エコー域が内部に認められた.

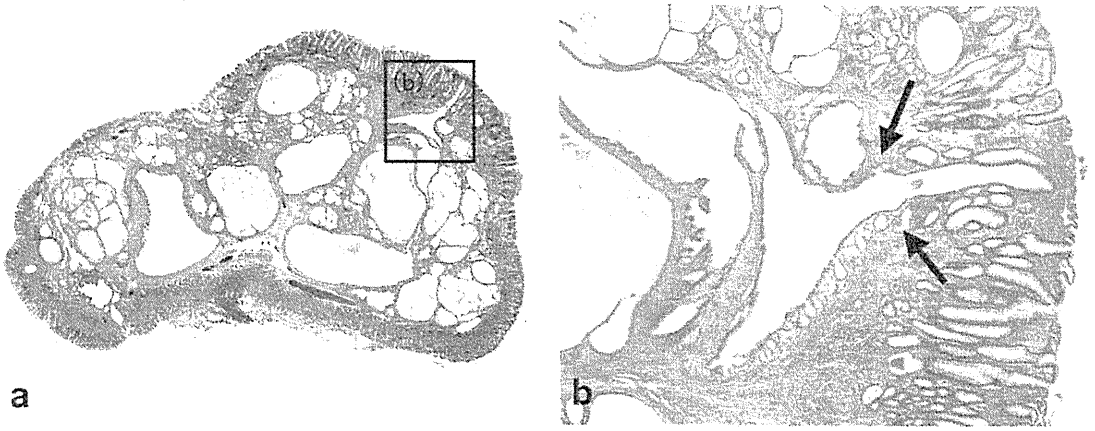

Figure 8 症例2の病理組織学的所見.
a：粘膜下層に多房性嚢胞状に拡張した腺管群と，それをとり囲む線維筋成分の増生からなる腫瘤病変を認めた（HE染色ルーペ像）.
b：一部粘膜筋板が欠落した部分で腫瘤を被覆する胃粘膜上皮が落ち込むようにして粘膜下の拡張した腺管につながる像が見られた（HE染色×40倍）.

(Figure 8-b). 以上より胃のHIPと診断した.

Ⅳ 考 按

胃の粘膜下異所腺の病態は大きく2つに大別することができる．1つは“びまん型”と言われるもので，胃底腺腺と幽門腺の境界領域に好発し，丈の低い比較的小さな粘膜下隆起が多発する病変であり，びまん性胃粘膜下異所腺や粘膜下異所性嚢胞と称されている[1),2)]．その98%に胃癌の併存を認めたとの報告もあり[1)]，Paracancerous lesionとして[2)]，臨床的にも問題とされる．これに対して“孤立型”の粘膜下異所腺病変は，胃体上部や胃穹窿部など胃底腺領域に好発し，比較的大きな単発性腫瘤をつくる傾向があり，胃過誤腫性ポリープやHIPと呼称されている．しかしながら，本来，先天性でneoplasmとしての意味合いの強い“過誤腫”という言葉を名称に含めることは，この病変には望ましくないとする考え方もあり，過去報告例には，過形成（hyperplastic）[3)]，もしくは単なるheterotopia[4)-7)]として表現されているものもある．臨床的に癌化[8)]もしくは癌併存[9)]報告は稀で，“びまん型”とは異なる病態と考えられている．

Table 1　本邦報告例（1989～2010）.

報告者	報告年	年齢	性別	症状	病変部位	大きさmm	形態	EUS所見	筋板の欠損と粘膜の落ち込み像	術前診断	治療
加藤ら[11]	1989	76	男	なし	前庭部	32×20×25	SMT	記載なし	記載なし	記載なし	開腹手術
瀧澤ら[12]	1990	48	女	なし	体上部	25×20×15	SMT	3層主体の大小の無エコー域	記載なし	過誤腫	開腹手術
杉山ら[5]	1992	15	女	心窩部痛	体上部	10×9×8	SMT	3層主体のびまん性高エコー	あり	記載なし	polypectomy
加藤ら[13]	1993	72	男	貧血	体上部	10	SMT	3層主体の大小不同無エコー域	あり	胃嚢胞	strip biopsy
					体中部	10	SMT	3層主体の大小不同無エコー域	あり	胃嚢胞	strip biopsy
松岡ら[14]	1995	39	女	なし	穹窿部	18×17	polyp	多発性の無エコー域	記載なし	過誤腫様ポリープ	polypectomy
井上ら[7]	1995	67	男	なし	体上部	10×9×5	polyp	3層主体の多房性無エコー域	あり	胃粘膜下異所腺	polypectomy
多田ら[15]	1996	68	男	体重減少	噴門部	70×60×40	polyp	大小不同の無エコー域	あり	記載なし	開腹手術
由際ら[8]	1997	61	男	貧血	前庭部	15	polyp	記載なし	あり	記載なし	polypectomy
		50	男	心窩部不快	前庭部	20	polyp	記載なし	あり	記載なし	polypectomy
		75	男	なし	前庭部	10	polyp	記載なし	あり	記載なし	polypectomy
加藤ら[16]	1997	57	男	なし	体上部	32×27×20	polyp	記載なし	あり	過形成性ポリープ	polypectomy
由際ら[17]	1998	8	女	下痢，嘔吐	前庭部	20	SMT	記載なし	あり	記載なし	開腹手術
Itoh ら[9]	1998	41	女	心窩部不快	穹窿部	23×18×9	polyp	記載なし	あり	記載なし	polypectomy
小沢ら[18]	2000	40	女	なし	穹窿部	25×17×9	SMT	3層主体の多房性無エコー域	あり	HIP，リンパ管腫	polypectomy
Kubo ら[19]	2000	21	女	消化管出血	穹窿部	記載なし	polyp	3層主体の多房性無エコー域	記載なし	記載なし	polypectomy
日比野ら[20]	2002	63	男	なし	穹窿部	28×25	SMT	3層主体の数個の無エコー域	記載なし		開腹手術
梅岡ら[21]	2003	41	男	消化管出血	体上部	5×5	SMT	記載なし	記載なし	記載なし	開腹手術
Aoki ら[10]	2004	43	女	なし	体上部	28	SMT	3層主体の不均一な低エコー腫瘍	あり	記載なし	開腹手術
平崎ら[6]	2006	39	女	なし	体上部	17×12	SMT	2，3層主体の低エコー	記載なし	迷入膵	開腹手術
Odashima ら[22]	2008	37	男	なし	穹窿部	25	SMT	3層主体の不均一な低エコー腫瘍	あり	記載なし	全周切開＋EMR
岡本ら[23]	2010	59	女	なし	体上部	18×15	polyp	3層主体に充実部と大小の嚢胞	あり	HIP	polypectomy
友松ら[24]	2010	48	男	なし	体下部	記載なし	SMT	単房性の無エコー域	あり	HIP	ESD
横沢ら[25]	2010	74	女	心窩部不快	体上部	20×17	polyp	3層主体の多房性無エコー域	記載なし	記載なし	polypectomy
自検例	2011	74	男	心窩部不快	穹窿部	18×16	SMT	3層主体の多房性無エコー域	あり	HIP	ESD
		61	女	なし	体上部	15×10	polyp	3層主体の多房性無エコー域	あり	HIP	polypectomy

Aokiらはこの HIP を更に，茎を有する polyp type と無茎性の SMT type に分類している[10]．Aokiらの分類方法に従うと，自験例1は明らかな茎がないことより SMT type で，自験例2は亜有茎性であることより polyp type に相当する．

HIP の術前診断が難しい理由として，その頻度の低さがある．HIP あるいは過誤腫をキーワードに医学中央雑誌，PubMed および引用文献より，1989年～2010年の21年間で抄録を除き検索した範囲では自検例を含め，本邦報告例は25例26病変であった（Table 1）[4), 5), 7)～25)]．このように稀な病変のため，内視鏡医が遭遇する頻度は極めて低いと思われる．また粘膜下に主座をおく病変であるため，生検での確定診断が難しいと思われる．これに対し，岡本らは内視鏡的にびらん，発赤および陥凹局面といった表面変化が，過去報告例の72.7%に認められており，これらの所見は粘膜下の病変本体が表面に開口しかけた部分を見ている可能性があり，HIP の内視鏡的診断に有用な所見として挙げている[23]．しかし自験例ではこれらの所見は明らかでなかった．

EUS が HIP の診断に有用であるとの報告がなされている[7), 14)]．典型例では HIP に特徴的な粘膜下層に嚢胞状に拡張した腺管構造が，第3層を主座におく多房性無エコー域として描出される[7), 12)～15), 18)～20), 23)～25)]．しかしながら，非典型例では単房性の嚢胞からなる HIP の報告もあり，注意が必要である[24)]．術前の確定診断が難しいとされる HIP だが[24)]，自験例は2例とも，HIP に特徴的な EUS 所見が見られたことより，確診に至ることができた．

HIP は治療に関しては，これまで開腹手術または内視鏡による切除の報告がある．開腹手術例は特に Aoki らの分類での SMT type 症例が多く[9)～12), 17), 20), 21)]，逆に polyp type には内視鏡的ポリペクトミー切除が行われていることが多い[4), 5), 7), 8), 14), 17), 25)]．最近の報告では，SMT type の HIP に対して新たな治療法として ESD 加療を行った1報告がある[24)]．自検例1は，明らかな茎がなく，内視鏡的ポリペクトミーでは不完全切除となり得る可能性があり，ESD にて一括切除を行った．ESD は筋層直上を剝離していくため，粘膜下層を主座とする HIP でも取り残す可能性が低く，術後詳細な病理学的検討が可能となり，今後 SMT type の HIP に有用な治療法となりうると考えた．

HIP の発生機序についてはいまだ議論の余地がある．1996年に Allen らは直腸において大腸粘膜腺管が粘膜下層に迷入し嚢胞状増生し，腸管内に突出した病変を HIP of the rectum と報告した[26)]．これらはその組織所見と若年者に多く見られる点から，先天的な異常疾患として位置づけられている．胃の HIP はこの counterpart ととらえられているが，過去報告例の年齢中央値は48歳[23)]と高く，若年者での発生が少ないことより，後天的な異常との見方が現在の主流とされる．後天的な炎症／再生性変化の中で，粘膜筋板の間隙や欠損部から粘膜下層に落ち込んだ再生粘膜が増生して生じるとする考えもある[4), 6)]．自験例は2例とも，粘膜筋板が欠落した部分で粘膜上皮が落ち込むように粘膜下の拡張した腺管に移行する所見を認め，HIP の発生機序を考える上で興味深い所見と思われる．同様な所見は過去報告例の大多数に見られ[1)～8), 10), 13), 15)～18), 22)～24)]（Table 1），後天説を後押しする興味深い所見と考える．

V 結 語

特徴的な EUS 所見より診断し，内視鏡的に切除し得た胃 Hamartomatous inverted polyp の2例を報告した．HIP は稀な疾患であるが，内視鏡医として，粘膜下腫瘍の鑑別診断に考慮すべき疾患である．

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論文受付　平成23年1月10日
同　受理　平成23年6月15日

Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2012 | 120 | 752–764 doi: 10.1111/j.1471-4159.2011.07617.x

ORIGINAL ARTICLE

A novel anti-inflammatory role for spleen-derived interleukin-10 in obesity-induced hypothalamic inflammation

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Abstract

Obesity can be associated with systemic low-grade inflammation that contributes to obesity-related metabolic disorders. Recent studies raise the possibility that hypothalamic inflammation contributes to the pathogenesis of diet-induced obesity (DIO), while another study reported that obesity decreases the expression of pro-inflammatory cytokines in spleen. The following study examines the hypothesis that obesity suppresses the splenic synthesis of the anti-inflammatory cytokine, interleukin (IL)-10, thereby resulting in chronic hypothalamic inflammation. The results showed that due to oxidative stress or apoptosis, the synthesis of splenic IL-10 was decreased in DIO when compared with non-obesity rats. Splenectomy (SPX) accelerated DIO-induced inflammatory responses in the hypothalamus. Interestingly, SPX suppressed the DIO-induced increases in food intake and body weight and led to a hypothalamic pro-inflammatory state that was similar to that produced by DIO, indicating that hypothalamic inflammation exerts a dual effect on energy metabolism. These SPX-induced changes were inhibited by the systemic administration of IL-10. Moreover, SPX had no effect on hypothalamic inflammatory responses in IL-10-deficient mice. These data suggest that spleen-derived IL-10 plays an important role in the prevention of hypothalamic inflammation and may be a therapeutic target for the treatment of obesity and hypothalamic inflammation.

Keywords: hypothalamic inflammation, IL-10, Obesity, spleen.

J. Neurochem. (2012) **120**, 752–764.

Recent studies suggest that obesity is accompanied by chronic systemic low-grade inflammation (Hottamisligil 2006). Indeed, institution of a high-fat diet (HF) results in tissue inflammation caused by recruitment and activation of macrophages, and subsequent local or systemic release of pro-inflammatory cytokines can cause insulin resistance (Odegaard and Chawla 2005; Shoelson *et al.* 2006). Furthermore, a HF increases the expression of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, in the hypothalamus, which suggests that hypothalamic inflammation contributes to the secondary effects of diet-induced obesity (De Souza *et al.* 2005). Although the mechanisms underlying obesity-associated inflammation in peripheral tissues, such as liver and white adipose tissue, are well characterized, much less is known about the pathogenesis of obesity-induced hypothalamic inflammation.

The hypothalamus plays a central role in regulating food intake. In addition of the satiety center (ventromedial hypothalamus, VMH) and feeding center (lateral hypothalamus, LH), the hypothalamic arcuate (ARC) and paraventricular nucleus (PVN) also participate in regulating feeding behavior. Various neuropeptides have been identified that either inhibit or stimulate feeding behavior (Morley 1987). For example, α-melanocyte-stimulating hormone (α-MSH)

Received July 14, 2011; revised manuscript received November 1, 2011; accepted December 2, 2011.

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Abbreviations used: 4-HNE, 4-hydroxynonenal; α-MSH, α-melanocyte-stimulating hormone; ARC, hypothalamic arcuate; CART, cocaine- and amphetamine-regulated transcript; HF, high-fat diet; IL, interleukin; IL-10KO, IL-10-deficient mice (LH, lateral hypothalamus; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; PVN, neuropeptide Y (NPYparaventricular nucleus; RQ, respiratory quotient; SPX, splenectomy; TNF, tumor necrosis factor; t-MH, tele-methylhistamine; TMN, tuberomammillary nucleus; VCO_2, carbon dioxide production; VMH, ventromedial hypothalamus; VO_2, oxygen consumption; WAT, white adipose tissue.

and cocaine- and amphetamine-regulated transcript (CART) are anorexigenic whereas neuropeptide Y (NPY) and orexin-A stimulate food intake (Stanley and Leibowitz 1985; Kristensen *et al.* 1998; Sakurai *et al.* 1998).

The spleen is the largest lymphoid organ in the body and plays an important role in host immune function and blood filtration via the removal and destruction of aged or damaged erythrocytes and other blood cells (Dameshek 1955). Splenic gene expression of pro-inflammatory cytokines, such as TNF-α and IL-6, is decreased in the setting of obesity (Lamas *et al.* 2004). In contrast, IL-10, which is synthesized within multiple organs, including the spleen, is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines. Large amounts of IL-10 are produced from activated B-cells that mature in the marginal zone of the spleen. Recent studies suggest that IL-10-producing B-cells play a regulatory role in suppressing harmful immune responses (Pestka *et al.* 2004).

Based on these findings, the present study investigated the hypothesis that obesity suppresses the synthesis of IL-10 in the spleen, thereby resulting in chronic hypothalamic inflammation and altering feeding-related neuropeptides in the hypothalamus.

Research design and methods

Animals

Male Sprague–Dawley rats (250–280 g; Seac Yoshitomi), male C57Bl/6J mice (wild-type mice, 22–25 g; KBT Oriental, Japan), and IL-10-deficient mice (IL-10KO mice, 002251-B6.129P2-Il10<tm1Cgn>/J, donated by Sandy Morse, Jackson Laboratories, Bar Harbor, Maine, USA) were housed in a room with daily illumination from 7 AM to 7 PM (12/12-h light/dark cycle) and maintained at Oita University. IL-10KO mice were maintained for backcrossing at our university. Polymerase chain reaction (PCR) primers of 5′-CCACACGCGTCACCTTAATA-3′ (mutant forward), 5′-GTTATTGTCTTCCCGGCTGT-3′ (wild type reverse) and 5′-CTTGCACTACCAAAGCCACA-3′ (common) were used for genotyping. All studies were conducted in accordance with the Oita University guidelines, based on the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All rats and mice were handled for 5 min each on four successive days to equilibrate their arousal levels before the experiment (Sakata 1982).

Experimental protocol

Experiment 1. Rats were assigned to one of two different groups ($n = 6$ in each group) as follows: group 1, rats were fed a standard chow [Standard (20% fat, 56% carbohydrate, 24% protein; Clea Chow, Tokyo, Japan)] for 8 weeks and then treated with a sham operation (Sham); group 2, rats were fed Standard for 8 weeks and then underwent splenectomy (SPX). All rats were housed for an additional 4 weeks after completion of the interventions. At that time, anesthesia was induced with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg), the abdominal cavity was opened, and the spleen was carefully removed. For the Sham group, the abdomen was opened, but the spleen was not removed.

Experiment 2. Rats were assigned to one of three groups ($n = 6$ in each group) as follows: group 1, rats were fed Standard for 8 weeks and treated with Sham; group 2, rats were fed a HF (60% fat, 20% carbohydrate, 20% protein; Diet Research, New Brunswick, NJ, USA) for 8 weeks and treated with Sham, group 3: rats were fed a HF for 8 weeks and treated with SPX. For the subsequent 4 weeks, all rats were fed with Standard to calculate food intake over 24 h, and body weight was measured.

Experiment 3. Rats were assigned to one of four groups ($n = 6$ in each group) as follows: group 1, rats were fed a HF for 8 weeks after Sham and then given rat serum albumin for the following 4 weeks; group 2, rats were fed a HF for 8 weeks after SPX and then given rat serum albumin for the following 4 weeks; group 3, rats were fed a HF for 8 weeks after SPX and then given recombinant rat IL-10 (Wako Chemical, Osaka, Japan; 5 ng/day) for the following 4 weeks; group 4 (pair-fed group): rats were fed the amount of food consumed by the SPX-treated group for 8 weeks after Sham and then given rat serum albumin for the following 4 weeks. Osmotic pumps (Durect, Cupertino, CA, USA) containing rat serum albumin or recombinant rat IL-10 were implants in the back of all rats, parallel to the spine, for 4 weeks. The dose of recombinant rat IL-10 was determined by multiplying the average normal serum concentration of IL-10 (30 pg/mL) by the average total blood volume of 500 g rat. Food intake over 24 h was calculated by weighing the remaining food, and body weight was measured between 5 PM and 6 PM every day.

Experiment 4. C57Bl/6J and IL-10KO mice were both assigned to one of three groups ($n = 6$ in each group) as follows: group 1, mice were fed a HF for 8 weeks after Sham and administered mouse serum albumin for the subsequent 4 weeks; group 2, mice were fed a HF for 8 weeks after SPX and administered mouse serum albumin for the subsequent 4 weeks; group 3, mice were fed a HF for 8 weeks after SPX and administered recombinant mice IL-10 (Wako Chemical; 0.5 ng/day) for the subsequent 4 weeks. Food intake over 24 h was calculated by weighing the remaining food, and body weight was measured between 5 PM and 6 PM. Osmotic pumps (Durect) containing mouse serum albumin or recombinant mouse IL-10 (0.5 ng/day) were implants in the back of all mice, parallel to the spine, for 4 weeks.

All rats and mice were anesthetized with sodium pentobarbital (50 mg/kg i.p.) before being exsanguinated by transcardiac perfusion with 100 mL saline that contained 200 units of heparin.

Assessing splenic function

Splenic function was assessed by determining the percentage of abnormal erythrocytes (erythrocytes with membrane abnormalities visible under a light microscope) and Howell–Jolly bodies on a peripheral smear. The percentage of abnormal erythrocytes and Howell–Jolly body-containing cells among a minimum of 1000 erythrocytes in Romanowsky-stained peripheral blood smears was used as a measure of splenic function.

Splenic apoptosis assay

Apoptosis was determined by the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay using an *in situ* apoptosis

detection kit (Boehringer Mannheim, Ingelheim, Germany). Tissues were counter-stained with 4′,6-diamidino-2-phenylindole to visualize the nuclei. The percentage of cells in apoptosis was calculated as the number of transferase dUTP nick-end labeling -positive cells × 100/ total number of nucleated cells in the white pulp of the spleen.

Cytokine levels in the spleen, hypothalamus, and serum

Commercial ELISA kits (Invitrogen, Carlsbad, CA, USA) were used to measure TNF-α, IL-1β, monocyte chemotactic protein-1 (MCP-1), and IL-10 levels in the spleen, hypothalamus, and serum. Protein concentrations of each organ solution were analyzed using the Lowry method. The IL-10/TNF-α ratio was determined mathematically.

Measurement of α-MSH, CART, NPY and orexin-A content, and measurement of neuronal histamine turnover in discrete hypothalamic nuclei

Sections containing the LH, PVN, VMH and ARC were dissected with a tissue punch, at the appropriate levels, based on the rat brain map (Paxinos and Watson 1997). Brains were sectioned at 0, 2 and 4 mm relative to the anterior commissure. The PVN and VMH were removed from the 0 and 2 mm slices, and the LH and ARC were removed from the 2 and 4 mm slices. Rat enzyme immunoassay (EIA) kits (Phoenix Pharmaceuticals, Belmont, CA, USA) were used to detect levels of α-MSH, CART(61-102), NPY and orexin-A in each hypothalamic nucleus.

The major metabolic breakdown pathway of histamine in the brain is transmethylation of histamine into tele-methylhistamine (t-MH) by histamine *N*-methyltransferase and subsequent deamination by monoamine oxidase B. Pre-treatment with pargyline, an inhibitor of monoamine oxidase B, induces t-MH accumulation in the extraneuronal space as a major neuronal histamine metabolite. Sections containing tuberomammillary nucleus (TMN) were dissected with a frozen razorblade, at the appropriate levels, based on the rat brain map (Paxinos and Watson 1997). The t-MH content of each nucleus, including the TMN, was measured by a method described previously. This was done because histamine neurons are localized in the TMN and project to many areas, such as the PVN and the VMH (Oishi *et al.* 1987).

Western blotting

Frozen-tissue preparations were homogenized with sample buffer, centrifuged, and boiled. Total protein concentration of the tissue was quantified using the Bradford method. Equal amounts of total protein were loaded on 8% sodium dodecyl sulfate–polyacrylamide gels for electrophoresis and then electrophoretically transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk for 1 h, incubated overnight with primary antibodies at 4°C, and then incubated with the secondary antibody for 1 h at 20°C. The primary antibody solution consisted of polyclonal antiserum with specificity for rabbit CD20 (Reprokine, Rehovot, Israel), rabbit 4-hydroxynonenal (4-HNE; Enzo Life Science, Farmingdale, NY, USA), rabbit caspase 3 (Acris Antibodies GmbH, Herford, Germany), and rabbit Iba-1, a specific marker of microglia (Wako Chemical). CD20, 4-HNE, caspase 3 and Iba-1 were detected by enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL, USA) and quantified using Quantity One imaging software (Bio-Rad). Iba-1 expression was also examined in discrete hypothalamic nuclei.

Histological and immunohistochemical analyses

Spleen and brain samples were fixed in 4% buffered paraformaldehyde. For immunohistochemical staining of CD20 and Iba-1, 5-μm thick sections of spleen and brain were incubated with primary antibodies overnight at 4°C with rabbit anti-CD20 antibody (Reprokine) for rat spleen or with rabbit anti-Iba-1 antibody (Wako Chemical) for rat and mouse brain. Slides were subsequently washed with phosphate-buffered saline and incubated with biotin-conjugated goat anti-rabbit IgG (ABC reagent; Vector Laboratories, Burlingame, CA, USA). The immunoreactivity of each sample was visualized with diamino-benzidine (Nacalai Tesque, Kyoto, Japan).

For 4-HNE protein staining, 5-μm-thick spleen sections were incubated overnight at 4°C with rabbit anti-4-HNE antibody (Enzo Life Sciences) and then incubated with biotin-conjugated goat anti-rabbit IgG (ABC reagent; Vector Laboratories). Samples were visualized with rhodamine-conjugated streptavidin and counter-stained with 4′,6-diamidino-2-phenylindole to visualize the nuclei. Additionally, normal rabbit serum instead of aforementioned antibodies was used and a further incubation with secondary antibody was performed as a negative control. These tests resulted in negative staining.

Measurement of oxygen consumption

Oxygen consumption was calculated using an indirect calorimetry system (Oxymax; Columbus Instruments, Columbus, OH, USA). After the system was calibrated against standard gas mixtures, rats were placed in individual acrylic calorimeter chambers, with free access to food and water. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured during a 24-h period at 20°C. The respiratory quotient (RQ) is the ratio of the VCO_2 to the VO_2. Total VO_2 and VCO_2 during 24 h were determined by integrating areas under the VO_2 and VCO_2 curves (AUC VO_2 and AUC VCO_2) according to the trapezoidal rule. The area under the RQ curve (AUC RQ) was also calculated.

Statistics

Results were expressed as mean ± SEM. Statistical tests included two-tailed Student's *t*-test and 2-way ANOVA followed by Scheffe's test for *post hoc* comparison. For all tests, the level of significance was set at $p < 0.05$.

Results

HF causes splenic dysfunction

To determine whether obesity leads to splenic dysfunction, abnormal erythrocytes were quantified in both Standard and HF groups using a blood smear (Fig. 1a). The ability of the spleen to filter out dysfunctional red blood cells was first recognized as a splenic function by observing abnormal erythrocytes and the Howell–Jolly bodies on the surface of red blood cells (Dameshek 1955). The percentage of abnormal erythrocytes (standard 0.47 ± 0.39% vs. HF 2.31 ± 0.46%) and Howell–Jolly body formation was higher in the HF group than in the Standard group (Fig. 1b and c).

CD20 is a B-cell-specific molecule expressed on the cell surface. Immunohistochemistry demonstrated that CD20-

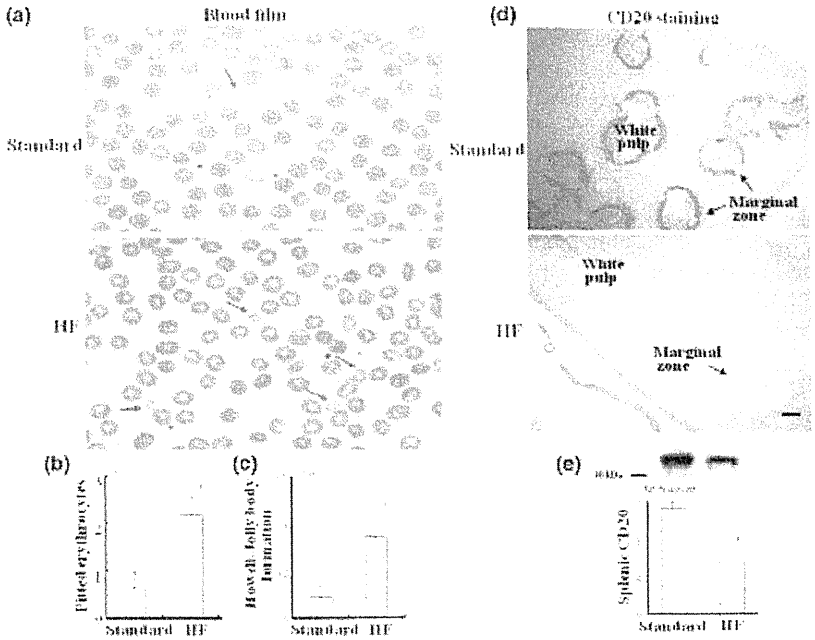

Fig. 1 HF-induced obesity leads to splenic dysfunction. (a) Representative red blood cell smears with abnormal erythrocytes (green arrow) from the Standard and HF groups. (b, c) Percentage of abnormal erythrocytes (b) and Howell–Jolly body formation (c) in each group ($n = 6$). (d) Representative CD20 staining (brown) in the marginal zone of spleen sections. Scale bar = 250 μm. (e) CD20 expression in the spleen in each group ($n = 6$). $^*p < 0.05$ versus Standard group. Treatment groups: Standard; standard chow, HF; high-fat diet.

positive areas of white pulp, including the marginal zone, were atrophied in the HF group (Fig. 1d) and that CD20 expression was decreased in the HF group when compared with Standard group (Fig. 1e).

HF induces oxidative stress and apoptosis in the spleen and modulates splenic and serum levels of pro- and anti-inflammatory cytokines

To examine the mechanisms underlying splenic dysfunction, oxidative stress and apoptosis were investigated in the spleen based on previous data that indicated that cadmium, which potentiates oxidative stress and activates the mitochondrial caspase-dependent apoptotic pathway, diminishes the size of white pulp in the spleen (Pathak and Khandelwal 2007). Levels of 4-HNE, a marker of oxidative stress, were significantly elevated in the marginal zone of the HF group when compared with Standard group, as illustrated by morphological (Fig. 2a) and semi-quantitative analyses (Fig. 2b). Additionally, there was an increase in apoptosis percentage of marginal zone area (Fig. 2c and d) and caspase 3 activity (Fig. 2e) in the HF group as compared with Standard group. These findings imply that HF-induced obesity leads to splenic dysfunction by promoting oxidative stress and apoptosis in the spleen. Despite the observation that the expression of TNF-α, IL-1β, MCP-1 and IL-10 in the spleen of HF group was significantly reduced, serum levels of IL-10, but not TNF-α, IL-1β, or MCP-1, were significantly decreased in the HF group when compared with Standard group (Fig. 2f and g). Serum cytokine levels, except IL-10, are probably maintained by induction from other organs, such as white adipose tissue (WAT) and liver, when the expression of splenic cytokines is down-regulated by HF feeding. However, serum IL-10 levels remained low, suggesting that large amounts of serum IL-10 are derived from the spleen.

Effect of SPX on splenic and serum levels of pro- and anti-inflammatory cytokines

To clarify the role of spleen in hypothalamic inflammation, it was investigated whether SPX aggravates local inflammation in the hypothalamus. Microglial activation was examined along with the expression of hypothalamic cytokines because the microglia is one of immune cells that synthesize both pro- and anti-inflammatory cytokines in the brain. The expression of Iba-1, a specific marker of microglia, in each nucleus (Fig. 3a and b) and the levels of pro- and anti-inflammatory cytokines in the hypothalamus were higher in Standard-fed SPX subjects than in Standard-fed Sham subjects (Fig. 3c). The IL-10/TNF-α ratio was also evaluated, because this ratio has been adopted as a marker of the intensity of the inflammatory condition in obese individuals, and because lower values are related to worse prognoses (Kaur *et al.* 2006; Jung *et al.* 2008). The hypothalamic IL-10/TNF-α ratio in was significantly lower in the SPX group than in the

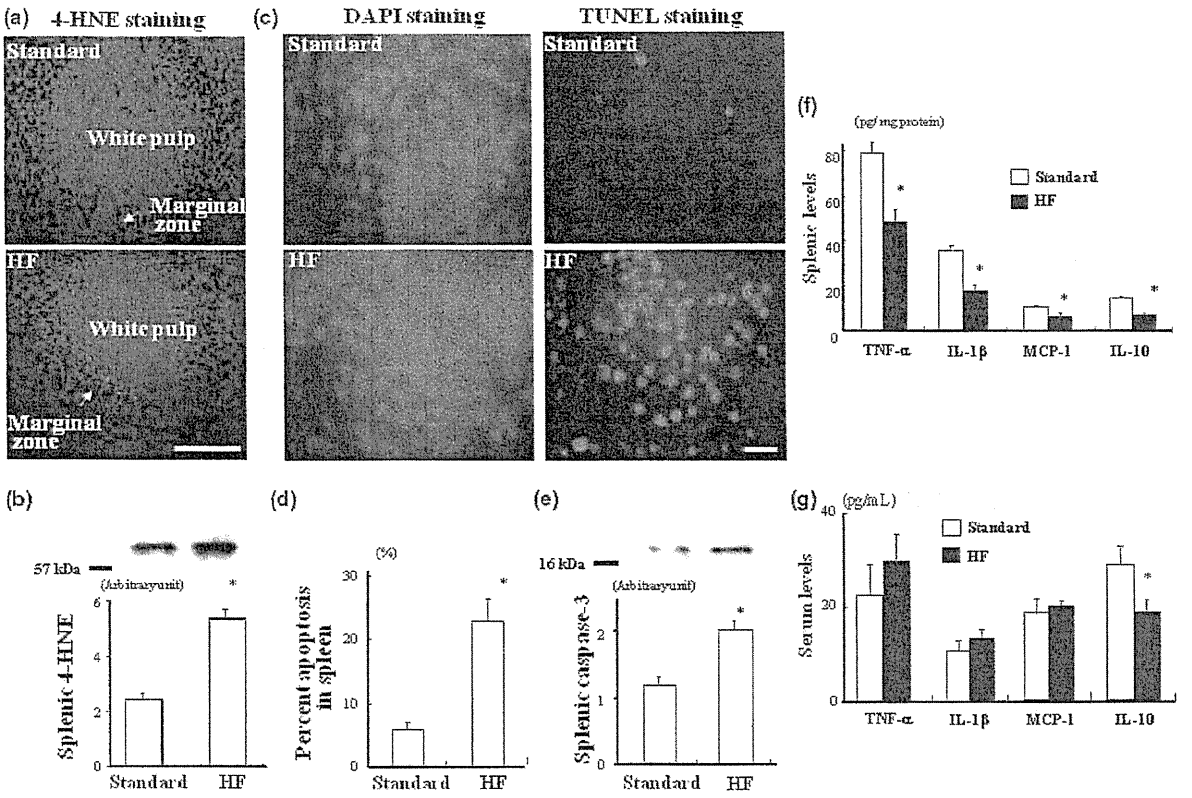

Fig. 2 HF-induced obesity causes oxidative stress and apoptosis in the spleen and decreases serum levels of IL-10 but not TNF-α, IL-1β and MCP-1. (a) Representative 4-hydroxynonenal (4-HNE) staining (red) counter-staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (blue) in the marginal zone of spleen sections. Scale bar = 250 μm. (b) 4-HNE expression in the spleen in each group (n = 6). (c) Representative DAPI (left row, blue) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL; right row, green; same sections as DAPI) staining. Scale bar = 20 μm. (d) Percentage of apoptotic (TUNEL+) cells in the marginal zone of the spleen in each group (n = 6). (e) Caspase 3 expression in the spleen in each group (n = 6). (f, g) Protein levels of TNF-α, IL-1β, MCP-1 and IL-10 in spleen (f) and serum (g) in each group. *p < 0.05 versus Standard group. Treatment groups: Standard; standard chow, HF; high-fat diet.

Sham group (Fig. 3d). Moreover, t-MH content in each nucleus (except for in the LH) was higher with SPX treatment than with Sham treatment (Fig. 3e). This metabolite of hypothalamic histamine is associated with an increased turnover of hypothalamic neuronal histamine, an anorexigenic monoamine. SPX treatment decreased food consumption and body weight significantly in comparison with Sham treatment (Fig. 3f and g). These findings suggest that SPX induces catabolism along with a decrease in production of hypothalamic cytokines. In addition, SPX treatment increased α-MSH levels in the PVN, VMH and ARC in comparison with Sham treatment. CART level was higher in the ARC, lower in the LH and unchanged in the PVN and VMH in SPX-treated animals when compared with Sham treatment (Fig. 3h and j). By contrast, orexin-A levels were lower in each nucleus as compared with Sham treatment, although there was no difference in NPY levels of each nucleus between SPX-treated and Sham-treated animals (Fig. 3k and l).

Effects of different magnitudes of inflammatory condition in the hypothalamus

To explore the possible role of the spleen in HF-induced hypothalamic inflammation, it was investigated whether SPX may aggravate HF-induced local inflammation in the hypothalamus. The hypothalamic expression of Iba-1 was higher in HF-fed Sham subjects than in Standard-fed Sham subjects but was lower in HF-fed Sham subjects than in SPX subjects (Fig. 4a and b). These findings indicate that splenic dysfunction or asplenia can prompt inflammation through the activation of microglia. In addition, the concentrations of pro- and anti-inflammatory cytokines in hypothalamus were increased in the HF-fed Sham group as compared with Standard-fed Sham group and were significantly elevated in the HF-fed SPX group with compared with HF-fed Sham group (Fig. 4c). The hypothalamic IL-10/TNF-α ratio in HF-fed Sham group was lower than in Standard-fed Sham group, and was significantly diminished in the HF-fed SPX group when compared with HF-fed Sham group, indicating that

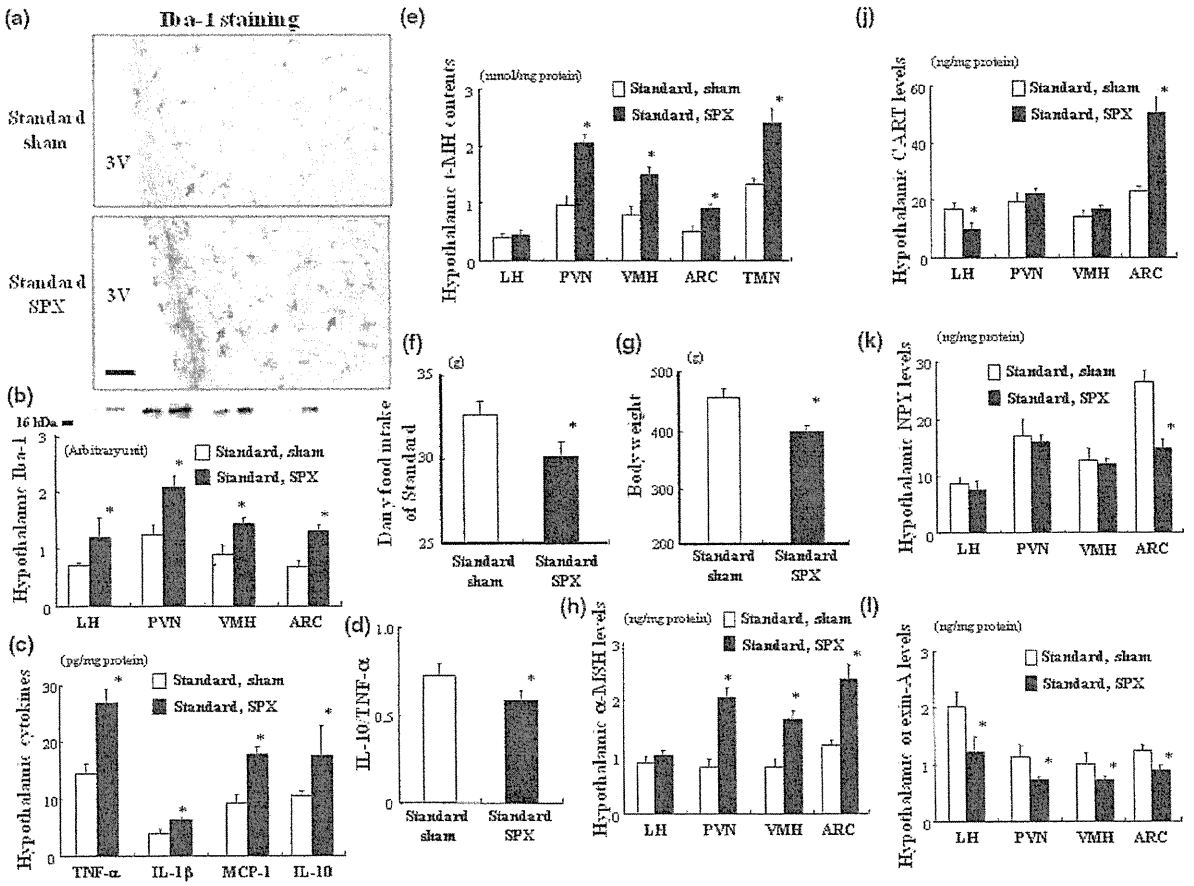

Fig. 3 Splenectomy accelerates inflammation in the hypothalamus and induces catabolic effects. (a) Representative Iba-1 staining (brown; green arrow) in hypothalamic sections. Scale bar = 20 μm. 3V; third ventricle. (b) Iba-1 expression in the hypothalamus in each group ($n = 6$). (c) TNF-α, IL-1β, MCP-1 and IL-10 contents in the hypothalamus in each group ($n = 6$). (d) The IL-10/TNF-α ratio in the hypothalamus in each group ($n = 6$). (e) t-MH contents in the hypothalamus in each group ($n = 6$). (f, g) Daily food intake of Standard (f) and body weight (g) in each group ($n = 6$). (h–l) Protein levels of α-MSH (h), CART (j), NPY (k) and orexin-A (l) in each nucleus of the hypothalamus ($n = 6$). *$p < 0.05$ versus Standard, Sham group. Treatment groups: Standard, Sham: fed a standard chow with a sham operation; Standard, SPX: fed a standard chow with a splenectomy.

SPX promotes obesity-induced hypothalamic inflammation (Fig. 4d). Moreover, hypothalamic t-MH content was higher in the HF-fed Sham group than in the Standard-fed Sham group but was significantly lower in the HF-fed Sham group than in the SPX group in each nucleus except for the LH (Fig. 4e). Despite the observation that SPX treatment leads to a pro-inflammatory state in the hypothalamus similarly to that induced by HF, SPX treatment significantly suppressed the increase in food consumption and body weight induced by HF, (Fig. 4f and g). These findings suggest that, depending on the intensity of local inflammation in hypothalamus, hypothalamic inflammation exerts a dual function, being highly catabolic in the presence of splenic dysfunction and being modestly anabolic in the context of HF-induced obesity. Furthermore, HF-fed treatment reduced α-MSH and CART levels in the PVN, VMH and ARC in comparison to Standard-fed treatment. SPX treatment increased α-MSH levels in the PVN, VMH and ARC whereas SPX increased CART only in the ARC as compared with Sham treatment. In addition, CART level in the LH was lower after SPX treatment, compared with Sham treatment (Fig. 4h and j). In contrast, HF-fed treatment increased NPY and orexin-A levels in the LH and ARC, and reduced NPY and orexin-A levels in the PVN and VMH, when compared with Standard-fed treatment. SPX treatment reduced orexin-A levels in each nucleus of the hypothalamus when compared with Sham treatment, although there was no significant alteration in the NPY level in each nucleus between Sham and SPX treatment (Fig. 4k and l).

IL-10 treatment inhibits SPX-induced catabolic effects

To explore the possible role of spleen-derived IL-10, the effect of recombinant IL-10 treatment on SPX-induced inflammation in hypothalamus was investigated. Correspond-

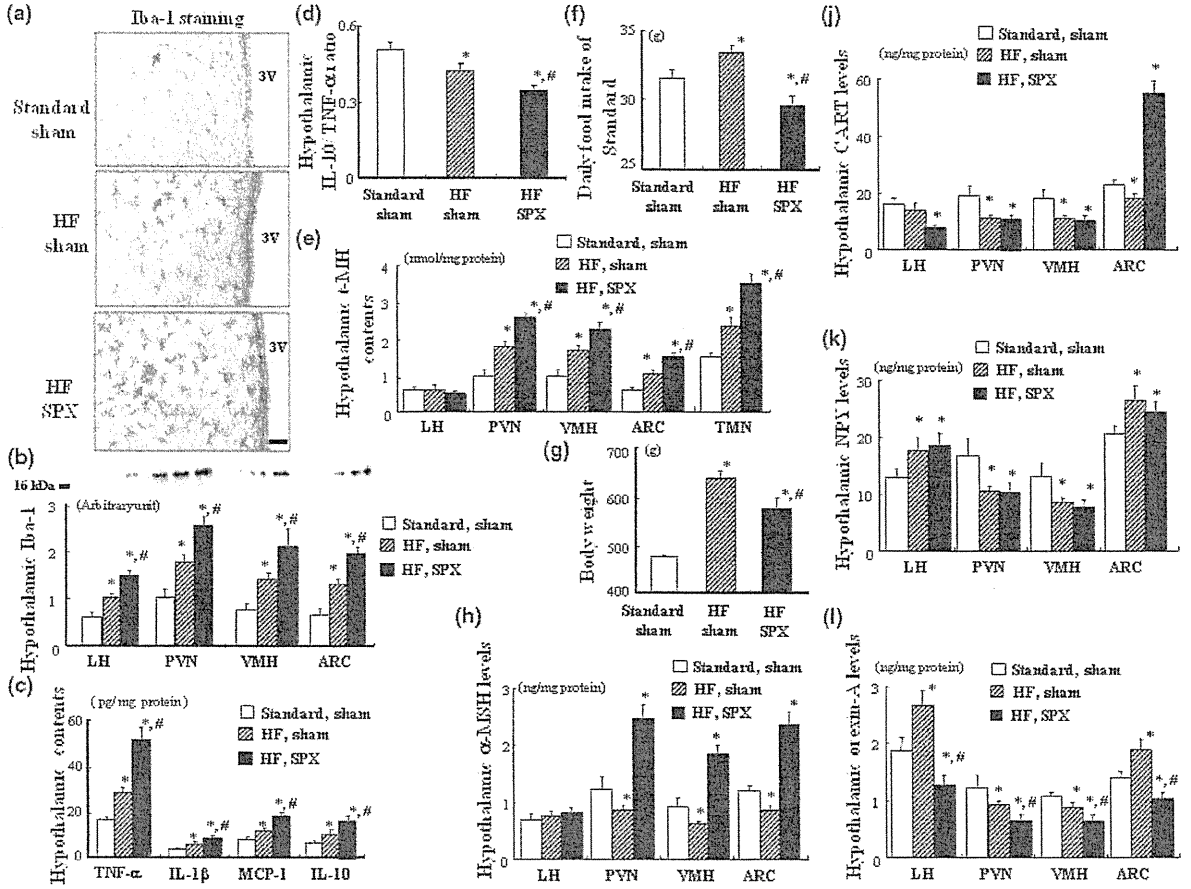

Fig. 4 Splenectomy promotes obesity-induced pro-inflammatory responses in the hypothalamus and induces anti-obesity effects. (a) Representative Iba-1 staining (brown; green arrow) in hypothalamic sections in each group. Scale bar = 20 μm. 3V; third ventricle. (b) Iba-1 expression in each nucleus of the hypothalamus ($n = 6$). (c) TNF-α, IL-1β, MCP-1 and IL-10 contents in the hypothalamus in each group ($n = 6$). (d) The IL-10/TNF-α ratio in the hypothalamus in each group ($n = 6$). (e) t-MH contents in each nucleus of the hypothalamus ($n = 6$). (f, g) Daily food intake of Standard (f) and body weight (g) in each group ($n = 6$). (h–l) Protein levels of α-MSH (h), CART (j), NPY (k) and orexin-A (l) in each nucleus of the hypothalamus ($n = 6$). $^{*}p < 0.05$ versus Standard, Sham group; $^{\#}p < 0.05$ versus HF, Sham group. Treatment groups: Standard, Sham; fed a standard chow with a sham operation, HF, Sham; fed a HF with a sham operation, HF, SPX; fed a HF with a splenectomy.

ing pair-fed treatment was used to assess the net effects of SPX on energy metabolism. IL-10 treatment abolished the SPX-induced decrease in food consumption of HF and weakened SPX-induced loss of body weight which was greater than that of the pair-fed treatment (Fig. 5a and b). Furthermore, IL-10 treatment abolished SPX-induced increase of VO_2 and VCO_2, and decrease of RQ (Fig. 5c–e). As a result, IL-10 treatment suppressed SPX-induced elevation of the AUC VO_2 and VCO_2, and reduction of the AUC RQ (Fig. 5f–h).

IL-10 treatment suppresses SPX-induced inflammatory responses in the hypothalamus

IL-10 treatment inhibited the SPX-induced increases in hypothalamic TNF-α, IL-1β, MCP-1, IL-10 (Fig. 6a), and t-MH content (Fig. 6c) as well as Iba-1 expression (Fig. 6d). IL-10 treatment also prevented the SPX-induced decrease in the hypothalamic IL-10/TNF-α ratio (Fig. 6b). Conversely, pair-fed treatment reduced expression of aforementioned cytokines, t-MH and Iba-1, and increased the ratio of IL-10/TNF-α in the hypothalamus when compared with HF-fed Sham treatment (Fig. 6a–d). Furthermore, IL-10 treatment inhibited the SPX-induced increase in α-MSH levels in the PVN, VMH and ARC, inhibited the SPX-induced increase in CART level in the ARC and decrease in that level in the LH, and repressed the SPX-induced decrease of orexin-A in each hypothalamic nucleus (Fig. 6e, f and h). IL-10 treatment had no effect on NPY levels in any of the nuclei (Fig. 6g). Meanwhile, the alterations of feeding-related neuropeptides mentioned above were reversed by pair-fed treatment except that the CART levels in the LH and PVN remained unchanged (Fig. 6e–h).

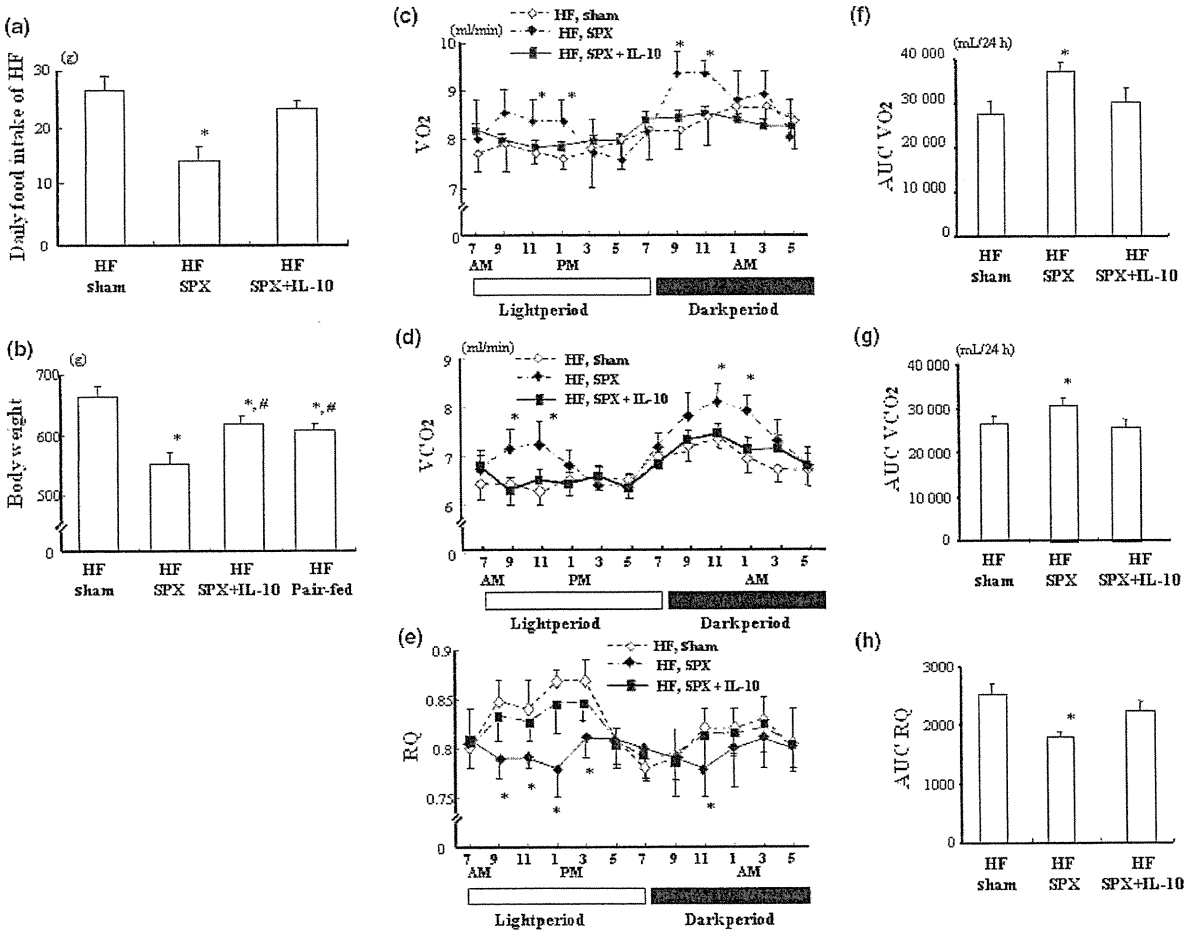

Fig. 5 Systemic administration of IL-10 suppresses splenectomy-induced catabolic effects. (a, b) Daily food intake of HF (a) and body weight (b) in each group ($n = 6$). (c–e) Daily monitoring of (c) oxygen consumption (VO_2), (d) carbon dioxide production (VCO_2), and (e) respiratory quotient (RQ) in each group ($n = 6$). (f–h) Area under the curves (AUC) of VO_2 (f), VCO_2 (g) and RQ (h) in each group ($n = 6$). $^*p < 0.05$ versus HF, Sham group, $^\#p < 0.05$ versus HF, SPX group. Treatment groups: Sham; serum albumin administration with a sham operation, SPX; serum albumin administration with a splenectomy, SPX + IL-10; IL-10 administration with a splenectomy, Pair-fed; serum albumin administration with a sham operation, fed the amount of food consumed by the SPX-treated group.

IL-10 deficiency reduces food intake and body weight, and SPX has little effect on inflammatory responses in the hypothalamus of IL-10 deficient mice

To further understand the influence of spleen-derived IL-10 protection, IL-10KO mice were used to determine whether IL-10 deficiency affects SPX-induced inflammation in the hypothalamus. IL-10KO mice fed with HF were growth retarded (Fig. 7a). In addition, food consumption and body weight significantly decreased in IL-10KO mice when compared with wild type mice (Fig. 7b and c).

The inflammatory condition in the hypothalamus was observed in IL-10KO mice (Fig. 7d and e), but SPX did not result in an increase in microglia expression or an increase in TNF-α, IL-1β, MCP-1 and t-MH levels (Fig. 7f–j) in the hypothalamus in IL-10KO mice. Similarly, SPX did not result in a reduction in food consumption of HF (Fig. 7k) or body weight (Fig. 7l) in IL-10KO mice. However, IL-10 treatment restored the factors mentioned above and increased food intake and body weight in both SPX-treated wild-type mice and in IL-10KO mice (Fig. 7f–l).

Discussion

Obesity is associated with insulin resistance, diabetes, dyslipidemia, and hypertension. Collectively, these conditions comprise metabolic syndrome, which is believed to involve a low-grade chronic pro-inflammatory state (Festa *et al.* 2001). However, the primary cause of obesity-induced inflammation is not well understood. This is the first study to characterize the impact of splenic dysfunction because of HF-induced obesity and SPX in rats with respect to the hypothalamus.

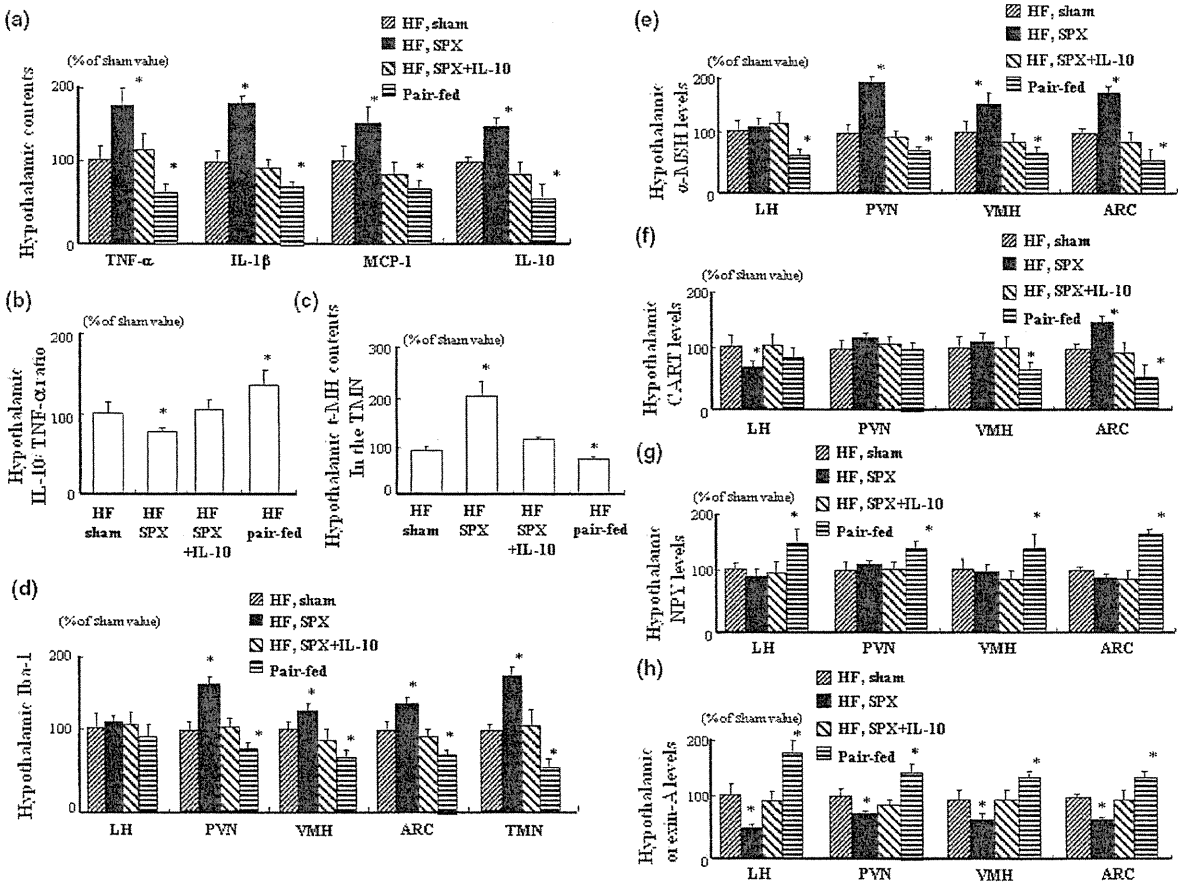

Fig. 6 Systemic administration of IL-10 suppresses splenectomy-induced pro-inflammation in the hypothalamus. (a) TNF-α, IL-1β, MCP-1 and IL-10 contents in the hypothalamus in each group shown in percentages relative to Sham values ($n = 6$). (b) The IL-10/TNF-α ratio in the hypothalamus in each group shown in percentages relative to Sham values ($n = 6$). (c) t-MH contents in the hypothalamus in each group shown in percentages relative to Sham values ($n = 6$). (d) Iba-1 expression in each nucleus of the hypothalamus shown in percentages relative to Sham values ($n = 6$). (e–h) Protein levels of α-MSH (e), CART (f), NPY (g) and orexin-A (h) in each nucleus of the hypothalamus ($n = 6$). *$p < 0.05$ versus HF, Sham group. Treatment groups: Sham; serum albumin administration with a sham operation, SPX; serum albumin administration with a splenectomy, SPX + IL-10; IL-10 administration with a splenectomy, Pair-fed; serum albumin administration with a sham operation, fed the amount of food consumed by the SPX-treated group.

Our research showed that HF feeding increased the percentage of abnormal erythrocytes in peripheral blood and down-regulated the expression of CD20, a surface molecule present on B-cells in the spleen. These results indicate that HF feeding reduces the ability of spleen to filter out abnormal erythrocytes and the expression of B-cells that play a large role in the immune response including IL-10 synthesis. The inability of the spleen to filter out abnormal erythrocytes and to synthesize cytokines is considered to represent splenic hypofunction. In addition, the present findings imply that HF-induced obesity leads to splenic hypofunction by promoting oxidative stress and apoptosis in the spleen, which is consistent with prior studies showing that the antioxidant, *N*-acetyl cysteine, attenuates lipopolysaccharide (LPS)-induced apoptosis in splenic B-cells (Martin *et al.* 2000).

Our results indicate that hypophagia and body weight loss after SPX treatment are caused by a SPX-induced hypothalamic inflammatory response associated with an increase in neuronal histamine and IL-1β expression, which is consistent with a previous finding that central administration of IL-1β activates histamine neuron and results in hypophagia (Kang *et al.* 1995). Microglia, which are the macrophages of the CNS, are activated by various cytokines, including metabolic hormones such as leptin, and produce IL-1β, TNF-α and IL-6 (Pinteaux *et al.* 2007; Tang *et al.* 2007; Lafrance *et al.* 2010). Hence, microglia could be involved in the aggravation of metabolic disorder caused by obesity-induced hypothalamic inflammation. Our results showing that SPX activated microglia in the hypothalamus and worsened HF feeding-induced inflammation in the hypothalamus suggest that

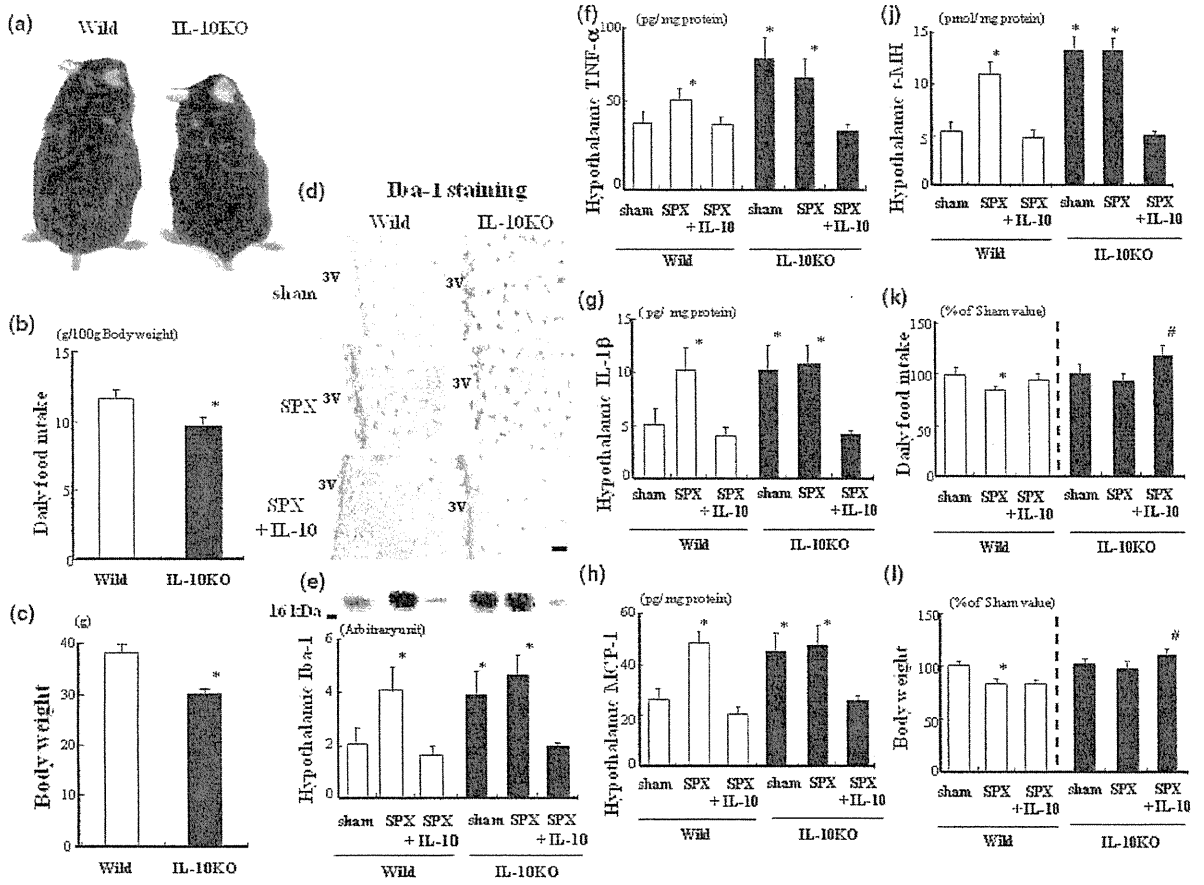

Fig. 7 IL-10 deficiency decreases food intake and body weight, and spleen-derived IL-10 participates in the regulation of hypothalamic inflammation and energy metabolism. (a) Appearances of growth in wild-type mice and IL-10KO mice fed HF. (b, c) Daily food intake of HF (b) and body weight (c) in each group ($n = 6$). $^*p < 0.05$ versus Wild. (d) Representative Iba-1 staining (brown) in the hypothalamus in each group. Scale bar = 20 μm. (e) Iba-1 expression in the hypothalamus in each group ($n = 6$). (f–h) TNF-α (f), IL-1β (g) and MCP-1 (h) contents in the hypothalamus in each group ($n = 6$). (j) t-MH contents in the hypothalamus in each group ($n = 6$). $^*p < 0.05$ versus Sham (Wild) group. (k, l) Daily food intake of HF (k) and body weight (l) of each group shown in percentages relative to Sham values ($n = 6$). $^*p < 0.05$ versus Sham (Wild) group, $^\#p < 0.05$ versus Sham (IL-10KO) group. Treatment groups: Sham; serum albumin administration with a sham operation, SPX; serum albumin administration with a splenectomy, SPX + IL-10; recombinant mouse IL-10 administration with a splenectomy, Wild; wild-type mice, IL-10KO; IL-10 deficiency mice.

microglia trigger inflammation in response to HF feeding, which induces splenic hypofunction.

IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines. IL-10 is synthesized within multiple organs, including the spleen. We focused on spleen-derived IL-10 because serum levels of IL-10, but not TNF-α, IL-1β, or MCP-1, were significantly decreased in the HF group compared with the Standard group, despite the finding that the expression of all cytokines in the spleen of the HF group were significantly reduced. This suggested that large amounts of serum IL-10 are synthesized in the spleen. Previous studies demonstrated that treatments that reduce Il-10 levels in diet-induced obese mice results in a progression of systemic inflammation and impairs insulin responsiveness (Cintra *et al.* 2008). Thus, it was hypothesized that the obesity-induced reduction in splenic IL-10 synthesis might lead to an inflammatory response in the hypothalamus and onset of metabolic disorders. In fact, obesity is associated with low IL-10 production capacity (Esposito *et al.* 2003; Waters *et al.* 2007). Moreover, we showed that IL-10 treatment reduced the expression of inflammatory cytokines and microglia in the hypothalamus, and improved the hypothalamic TNF-α/IL-10 ratio, indicating that IL-10 treatment attenuates the SPX-induced pro-inflammatory state in the hypothalamus. These observations are consistent with previous data that the administration of IL-10 attenuates inflammation-induced anorexia (Hollis *et al.* 2010). These findings indicate that splenic hypofunction can induce inflammation in the hypothalamus and that spleen-derived IL-10 may regulate the hypothalamic inflammatory response.

Inflammatory responses in the brain are mainly associated with microglia, which are the major immune effector cells in the CNS (Kreutzberg 1996; Block *et al.* 2007). IL-10 is produced by microglia in response to LPS treatment and brain injury, and IL-10 production suppresses the production of pro-inflammatory mediators, such as TNF-α and IL-1β from microglia (Mizuno *et al.* 1994). Considering our findings that hypothalamic IL-10 was endogenously elevated under SPX-induced hypothalamic inflammatory conditions and that IL-10 treatment restored this inflammatory response, IL-10 derived from spleen or the microglia themselves appear to be important anti-inflammatory modulators of glial activation, functioning to maintain a balance between pro- and anti-inflammatory cytokine levels in the hypothalamus. It is therefore possible that in glial cells, IL-10 acts in an autocrine or paracrine manner to down-regulate the synthesis of pro-inflammatory cytokines.

Furthermore, we evaluated neuropeptides involved in the control of feeding. Both α-MSH and NPY are synthesized in the ARC and project to the PVN, VMH and LH (Sahu *et al.* 1988; Mountjoy and Wong 1997; Elias *et al.* 1999; Cone 2005). CART is also mainly located in the ARC and is distributed in the PVN and VMH (Koylu *et al.* 1998). By contrast, orexin-A is mainly produced in the LH, and orexin-containing fibers are distributed throughout the PVN, VMH and ARC (Wang *et al.* 2003). This study showed that HF feeding accelerated hyperphagia, although all neuropeptides were decreased in the PVN and VMH, a satiety center. Appetite is controlled by a number of central mediators that may interact to regulate the influence of other systems on feeding behavior. It might be suggested that reduction of α-MSH and CART increases appetite, rather than reduction of NPY and orexin-A produces hypophagia. A previous study demonstrated that the peripheral LPS injection sufficient to elicit hypophagia caused (i) increases in mRNA levels of CART and pro-opiomelanocortin, which is cleaved into α-MSH; (ii) no increase in in NPY in the ARC; (iii) increases in α-MSH but not CART or NPY mRNA in the PVN; and (iv) prevented the activation of orexin neurons in the LH (Sergeyev *et al.* 2001; Becskei *et al.* 2008). These findings support our results that SPX led to an increase in α-MSH content in all nuclei except for the LH, an increase in CART content in the ARC, a decrease in CART content in the LH, no change in NPY content in any nuclei, and a decrease in orexin-A content in all nuclei. It is interesting to note that α-MSH and CART are co-localized in the ARC and that we observed a differential alteration between α-MSH and CART in the LH, PVN and VMH. This is consistent with a previous report showing a differential expression of pro-opiomelanocortin and CART in adrenalectomized rats (Vrang *et al.* 2003). Therefore, these data suggest the existence of distinct intracellular regulation of these neuropeptides.

As already mentioned, LPS induces microglia-mediated synthesis and release of pro-inflammatory cytokines in the brain. Previous research found that central injection of IL-10 attenuated the body weight loss induced by the central administration of LPS, which is consistent with the present result that IL-10 treatment abolished the SPX-induced body weight loss. This latter phenomenon was probably mediated by SPX-enhanced hypophagia and energy expenditure (Bluthé *et al.* 1999). Thus, we evaluated the effect of IL-10 on the alteration of hypothalamic neuropeptides induced by SPX. The present study revealed that α-MSH and orexin-A levels in each nucleus of the HF-fed SPX group returned to those of the HF-fed Sham group after treatment with exogenous IL-10, supporting the concept that spleen-derived IL-10 acts as an anti-inflammatory cytokine in the hypothalamus.

To further understand the influence of spleen-derived IL-10 protection, IL-10KO mice were used to determine whether IL-10 deficiency would affect SPX-induced inflammation in the hypothalamus. We observed that food intake and body weight were decreased in IL-10KO mice. This was probably caused by the inflammatory condition in the hypothalamus, as previous findings showed IL-10KO mice have spontaneous weight loss (Kühn *et al.* 1993). However, SPX-induced reduction in food intake, body weight, and pro-inflammatory responses were not seen in IL-10KO mice, despite the fact that IL-10 treatment increased food intake and body weight in SPX-treated IL-10 KO mice, and suppressed pro-inflammatory responses in both SPX-treated wild-type mice and IL-10KO mice. These results indicate that SPX has little effect on feeding behavior and hypothalamic inflammatory responses in IL-10KO mice and that spleen-derived IL-10 may affect the regulation of hypothalamic inflammation. We showed that the levels of inflammatory cytokines in the hypothalamus were increased in the obesity group when compared with control group, but were considerably lower in the obesity group than in the SPX-treated obesity group. Considering the finding that obesity causes a reduction in splenic IL-10 and that splenic IL-10 plays an important role in the prevention of hypothalamic inflammation, we suggest that spleen-derived IL-10 may be a therapeutic target in the management of the complications of obesity, including hypothalamic inflammation.

Hypothalamic inflammation may exert a paradoxical effect on energy metabolism. For example, hypothalamic inflammation induced by obesity results in hyperphagia and body weight gain, while hypothalamic inflammation induced in response to a systemic or local inflammatory process (e.g. bacterial sepsis) results in anorexia and weight loss. Prior studies have demonstrated that, in the context of sepsis, IL-10 is produced mainly by peritoneal neutrophils, while splenic leukocytes produce comparatively little IL-10 (Kasten *et al.* 2010). We demonstrated that a reduction of spleen-derived IL-10 resulted in activation of microglia and induction of hypothalamic inflammation, which may explain why anorexia and body weight occur in the setting of

sepsis-induced hypothalamic inflammation. Furthermore, we also found that hypothalamic inflammation leads to catabolism after SPX, but results in anabolism in the context of obesity. A recent study showed that TNF-α can exert a dual effect in the hypothalamus, depending on the dose employed; central injection of high dose TNF-α had an anorexigenic effect, whereas central injection of low dose TNF-α had an orexigenic effect (Arruda *et al.* 2011). The present study may imply that mild reduction of spleen-derived IL-10 by HF feeding causes hyperphagia, whereas s severe reduction of splenic IL-10 by SPX causes hypophagia. Thus, it is assumed that the effects of energy metabolism change sharply with the differences in the IL-10/TNF-α ratio as well as the TNF-α level in the hypothalamus. A recent study demonstrated that intrahypothalamic infusion of recombinant IL-10 blocked IKK/NF-κB signaling and endoplasmic reticulum stress and restored Akt and STAT3 phosphorylation, promoting anti-obesity. This suggests that modulation of hypothalamic IL-10 expression could constitute a promising alternative to reduce hypothalamic inflammation and endoplasmic reticulum stress related to obesity (Ropelle *et al.* 2010). A plausible hypothesis to explain this paradox of the hypothalamic inflammation proposes that the ability to synthesize IL-10 from the spleen is the first target of HF-induced hypothalamic inflammation and that this paradox is a result of difference in the magnitude of IL-10 induction from the spleen.

Although additional work is needed to understand why obesity elicits inflammatory responses in the hypothalamus, the results of this study indicate that a more comprehensive understanding of the interactions between obesity and the spleen may help to identify new approaches for the prevention and treatment of obesity and metabolic syndrome.

Acknowledgements

This study was supported by a grant from the Japanese Ministry of Health for regulating energy metabolism. We state that all authors declare that none of the authors has a financial interest conflict related to this work.

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Hepatology Research

Hepatology Research 2012; 42: 203–212 doi: 10.1111/j.1872-034X.2011.00914.x

Original Article

Involvement of remnant spleen volume on the progression of steatohepatitis in diet-induced obese rats after a splenectomy

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Aim: This study investigated the correlation between remnant spleen volume after splenectomy (SPX) and the degree of hepatic steatosis and/or inflammation.

Methods: Male Sprague–Dawley rats were fed HF food and divided into three groups: sham-operation (Sham) group, a hemisplenectomy (H-SPX) group, and a total-splenectomy (T-SPX) group. Serum was collected and livers removed 12 weeks after surgery. We measured serum lipid markers and evaluated liver changes by comparing the three groups. Additionally, we examined liver changes 24 weeks after SPX.

Results: Serum triglyceride and free fatty acid levels after SPX were higher than those of sham controls, and a significant difference was found between T-SPX and the other groups ($P < 0.05$ for each). Increased intrahepatic fat accumulation was shown in SPX rats along with lower residual spleen volume; this fat accumulation after SPX was accelerated in rats at 24 weeks. Additionally, liver inflammatory changes, including an increase in the Kupffer cell population and pro-inflammatory cytokine production, as well as a high level of oxidative stress, were observed in the liver sections from SPX rats, which correlated significantly with less volume of the residual spleen. Also, an increase in pro-inflammatory cytokine content and a decrease in anti-inflammatory cytokine content were shown in the residual spleen from H-SPX rats, as compared to those of sham controls ($P < 0.05$ for each).

Conclusion: These results indicate the importance of preserving splenic tissue. This residual spleen may play an important role in preventing the progression from diet-induced hepatic steatosis to steatohepatitis.

INTRODUCTION

OBESITY IS A systemic low-grade inflammatory state associated with increased levels of plasma pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-β, and IL-6.[1–4] The incidence of obesity and subsequent activation of the immune system contribute to an increase in diet-induced hepatic steatosis that progresses to steatohepatitis and is involved in an inflammatory state of the liver.[5] These observations suggest that the immune system plays an important role in the progression of obesity-associated fatty liver disease and the development of steatohepatitis.

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Received 17 June 2011; revision 6 September 2011; accepted 11 September 2011.

The spleen is a large immunological organ that has an anatomical correlation with the liver and contains approximately 25% of all lymphocytes and up to 15% of fixed macrophages.[6] Several studies have been conducted using splenectomized rodents to investigate the role of the spleen in regulating the host immune system.[7–12] In addition to immunological functions, some reports considered the relationship between the spleen and lipid metabolism using splenectomized rodents fed a high-cholesterol or high-fat diet.[13–15] These reports indicate that a splenectomy (SPX) induces high levels of serum triglyceride (TG) and low-density lipoprotein (LDL), which increase the extent and severity of atherosclerosis.[13,14,16] These contributions of lipid metabolism after spleen removal were induced by two factors in these previous studies. One is a loss of splenic B cells and subsequent reduction in antibody production from splenic B cells toward autoantigens such as oxidized LDL. The other is a loss of splenic macrophages, which contain lipid fractions such as non-esterified

fatty acids and LDL. According to a report in humans, the changes in lipid metabolism caused by SPX due to trauma may eventually explain the high incidence of acute myocardial infarction detected in World War II veterans.[17] Taken together, the spleen may also play an important role in regulating systemic lipid metabolism in humans and rodents.

In a recent study, a total SPX has been suggested to affect steatohepatitis.[18] A total SPX produces a state with a lack of spleen tissue and complete loss of function, resulting in impaired splenic function. In previous reports, partially splenectomized animals exhibit a tendency to develop dyslipidemia similar to the changes caused by a total SPX.[13,15] Furthermore, Malangoni *et al.*[19] demonstrated that the phagocytic function of the spleen after a partial and total SPX correlates highly with the weight of the splenic remnant, indicating that a smaller splenic remnant causes impaired phagocytic function. Thus, we hypothesized that the hepatic and systemic effects of SPX are induced by impaired splenic function following depletion of spleen tissue but not complete loss of the organ. To address this hypothesis, this study was conducted to investigate the correlation between remnant spleen volume after SPX and the degree of hepatic steatosis and/or inflammation using total and partially splenectomized obese rats. We also evaluated the importance of preserving splenic function in obese animals.

METHODS

Animals and surgical procedures

MALE SPRAGUE-DAWLEY RATS (KBT Oriental, Fukuoka, Japan), 8 weeks of age, were housed in a light-, temperature-, and humidity-controlled room (12:12 h light : dark cycle with lights on/off at 0700/1900 hours; 21 ± 1 °C; 55 ± 5% relative humidity). The rats were allowed free access to chow pellets and water during the experiment. All animals were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals.

Experiment 1

Fifteen rats were fed 60% high-fat (HF) food (20% protein, 20% carbohydrate, and 60% fat; 5.2 kcal/g; Research Diet, Tokyo, Japan) for 4 weeks and then divided into three groups: Sham group (animals submitted to laparotomy with spleen manipulation; $n = 5$); H-SPX group (hemisplenectomy that preserved the upper splenic pole remnant; $n = 5$); T-SPX group (total splenectomy; $n = 5$).

Following an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and induction of deep anesthesia, total splenectomy (T-SPX) and hemisplenectomy (H-SPX) were aseptically performed through a left-sided lateral 1.5-cm subcostal incision. When T-SPX was assigned, the splenic vessels were ligated using 4-0 silk suture, and the spleen was removed. A splenic section line, at the middle of the spleen between the upper pole and lower pole, was identified first for the H-SPX, and then the spleen itself was ligated using 4-0 silk suture based on this line. Next, only lower pole vessels were ligated using 4-0 silk sutures. These vessels were cut, and the spleen was resected carefully at the splenic section line, preserving the upper pole and approximately the lower half of the entire spleen. The incision was closed in layers using running 4-0 nylon sutures. A sham operation was performed through the same incision, but the spleen was returned to the abdominal cavity without ligation, and the wound was closed. All animals were fed HF food for 12 weeks after splenic surgery.

Experiment 2

Twenty rats were fed 60% HF food for 4 weeks and then divided into two groups, Sham group (sham operation; $n = 10$) and SPX group (total splenectomy; $n = 10$). Same surgical procedures, both sham operation and total splenectomy, were performed as Experiment 1. After splenic surgery, each group was divided into two subgroups. One subgroup was fed the same HF food for 12 weeks (12W group), and the other was fed HF food for 24 weeks (24W group) after surgery.

Measurements and blood sampling

At the end of both experiments, the rats were anesthetized with sodium pentobarbital, as above, and perfused transcardially with isotonic PBS, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The liver and spleen were removed immediately, frozen in liquid nitrogen, and stored at −80 °C until protein extraction. In Experiment 1, blood was withdrawn via cardiac puncture, and the serum was separated and immediately frozen at −80 °C until analysis. Serum triglyceride (TG), free fatty acid (FFA), total cholesterol (TC) and alanine aminotransferase (ALT) was measured using an automatic analyzer (SRL, Tokyo, Japan).

Histological analysis and immunohistochemistry

Liver tissue samples were fixed with 10% formalin and embedded in paraffin. These sections of 5-μm thickness were cut with a Vibratome, and liver sections stained

with hematoxylin and eosin (H&E), and examined under a microscope (Olympus, Tokyo, Japan).

Immunohistochemistry was performed to stain Kupffer cells (KCs) using an antibody against CD68 (AbD Serotec, Oxford, UK). Liver tissue slices, cut from the samples described above, were washed three times in PBS, incubated for 1 h in 0.3% H_2O_2 to quench endogenous peroxidase activity, and transferred without rinsing to the primary antibody solution, which consisted of 5 g/L polyclonal anti-CD68 mouse antiserum. After 24 h at 4 °C, the slices were washed three times in PBS and processed using the ABC method (Vector Laboratories, Burlingame, CA, USA). The slices were transferred to a biotinylated anti-mouse antibody solution for 1 h, washed, transferred to avidin-biotinylated peroxidase for 1 h, washed, and then developed with diaminobenzidine substrate for 10 min. The slices were washed and mounted on slides with Permount.

Intrahepatic TG contents and cytokine levels in liver and spleen

Liver and spleen tissue (100 mg) was homogenized in 2 mL of a solution containing 150 mM NaCl, 0.1% Triton X-100, and 10 mM Tris, using a Polytron homogenizer (NS-310E; Micro Tech Nichion, Chiba, Japan) for 1 min. The liver TG contents were determined using a commercially available kit (Wako Chemical, Osaka, Japan). Commercial enzyme linked immunosorbent assay (ELISA) kits were used to measure the amounts of the cytokines TNF-α, IL-1β, monocyte chemotactic protein (MCP)-1 (Invitrogen, Camarillo, CA, USA) and IL-10 (R&D Systems, Minneapolis, MN, USA). Optical density readings of all samples were converted to pg/mL using standard curves generated with the recombinant cytokines supplied with the kits. Protein concentrations were determined using a protein assay kit (Bio-Rad

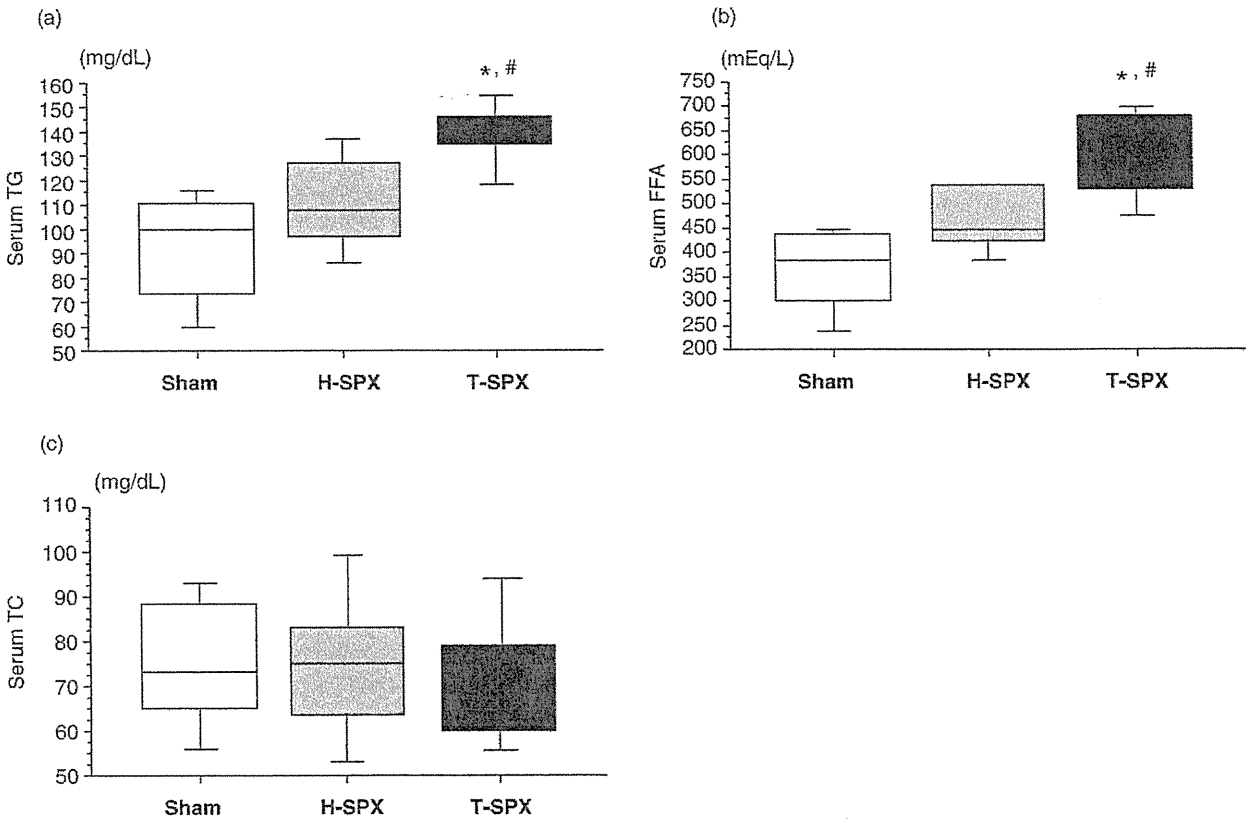

Figure 1 Systemic dyslipidemia was observed in splenectomized (SPX) rats. Serum was collected at 12 weeks after splenic surgery, and the levels of triglycerides (TG) (a), free fatty acids (FFA) (b), and total cholesterol (TC) (c) were measured. Data are shown as means ± standard error of the mean (SEM) ($n = 5$ rats/group). $^*P < 0.05$ *vs.* Sham, $^\#P < 0.05$ *vs.* hemisplenectomy (H-SPX).