された効率的な虚血性心疾患発症の相対リスク低下が，真に日本人はスタチンの多面的作用の恩恵を享受しやすいことを示唆しているのかという疑問に答えるため，今後さらに，日本人を対象にしたエビデンスを構築していくことが重要と考えられる．

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NO suppresses while peroxynitrite sustains NF-κB: a paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO

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Abstract

Objective: NO has both cytoprotective and cytotoxic effects. A key cytoprotective action of NO is attributed to inhibition of nuclear factor-κB (NF-κB)-mediated gene expression; this potentially endows NO with ubiquitous anti-inflammatory activity. Since immunostimulant-induced iNOS gene expression is itself dependent on NF-κB, NO is expected to limit its own synthesis. On the other hand, many cytotoxic actions of NO have been attributed to the chemical reactivity of peroxynitrite ($ONOO^-$) formed from NO by near diffusion-limited reaction with O_2^-. To assess whether $ONOO^-$ shares the ability of NO to inhibit NF-κB activation and consequent iNOS gene expression, we compared effects of NO donors (NOR3 and SNAP), an $ONOO^-$ donor (SIN-1), and pure $ONOO^-$ on LPS-induced responses in vascular smooth muscle cells (VSMC). **Methods and results:** NO donors, but not $ONOO^-$, suppressed LPS-induced NF-κB activation and expression of a murine iNOS promoter/reporter construct. An NO donor also suppressed NF-κB activation when induced by IL-1β or TNFα. Northern blot and RT-PCR analyses showed that NO, but not $ONOO^-$ or 8-bromo-cGMP, decreases LPS-induced expression of iNOS mRNA. Electrophoretic mobility shift assays (EMSA) and immunocytochemical analyses confirmed that NO but not $ONOO^-$ inhibits nuclear translocation of NF-κB. Although $ONOO^-$ generation from SIN-1 did not inhibit NF-κB activation, conversion of SIN-1 to a pure NO donor (by addition of excess superoxide dismutase) resulted in potent inhibition of NF-κB activation. Dose–response analyses suggest that the inhibitory effect of NO on iNOS gene transcription results specifically from inhibition of NF-κB activation, and is mediated by a G-cyclase-independent mechanism that is unavailable to $ONOO^-$. LPS stimulates IκB-α phosphorylation by inducing IκB kinase (IKK) activity, and NO, but not $ONOO^-$, inhibits LPS-induced IκB-α phosphorylation and IKK activity. **Conclusion:** We demonstrate that only NO inhibits the activation of NF-κB and suppresses iNOS gene expression. This distinction provides a novel paradigm to rationalize cytoprotective and cytotoxic actions attributed to NO.

Keywords: Nitric oxide; Peroxynitrite; Nuclear factor κB; IκB kinase; Vascular smooth muscle cells

1. Introduction

Endothelial-derived nitric oxide (NO) plays a physiological role in regulating vascular tone and blood pressure, maintaining a nonadhesive vasoluminal surface, mediating angiogenesis and preventing inappropriate proliferation of underlying vascular smooth muscle cells (VSMC). In contrast to these beneficial actions of constitutively produced NO, immunostimulants trigger expression of the inducible NO synthase (iNOS) gene product, leading to NO overproduction and vasopathophysiology [1]. iNOS-derived NO overproduction appears to be a ubiquitous mediator of vascular inflammatory conditions, including atherogenesis [2] and various forms of circulatory shock [3,4]. Importantly, the systemic inflammatory response is also defined by the production of oxygen-derived free radicals [5]. There is now substantial evidence that immune stimulus-evoked cytotoxicity involves the concerted action of reactive oxygen- and nitrogen-derived species. Indeed, a major component of injury is associated with the simultaneous production of NO and O_2^-, leading to near diffusion-limited production of the reactive species, peroxynitrite ($ONOO^-$) [6]. Synthesis of $ONOO^-$ has been considered as a channeling

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mechanism that diverts NO from physiological to pathophysiological targets. The ability of immunostimulants to upregulate iNOS gene expression in rodents [7] and man [8] is dependent on activation of the transcription factor, nuclear factor κB (NF-κB). Activated NF-κB serves as the master coordinator of the inflammatory response, binding to consensus elements in the promoters of diverse immunostimulant-inducible genes and upregulating transcription (for review, see Refs. [9,10]). Dormant NF-κB resides in the cytosol of quiescent cells bound to inhibitory proteins, isoforms of IκB. Inflammatory stimuli and oxidative stresses trigger rapid phosphorylation of IκB, resulting in targeted degradation by the proteasome. This results in untethered cytosolic NF-κB that undergoes nuclear translocation, followed by binding to promoter sequences in cognate target genes that elicit transcriptional upregulation. Recently, a cytokine-responsive IκB-α kinase (IKK) that activates NF-κB by phosphorylation of Ser32 and Ser36 residue in IκB-α has been identified [11–13]. In the murine iNOS gene, a 5′-flanking NF-κB binding element was shown to be both necessary and sufficient to initiate transcription in macrophages treated with bacterial lipopolysaccharide (LPS) [7]. In rat VSMC, LPS and interferon-γ (IFN) act synergistically to induce iNOS expression [14] by a process that is inhibited by the NF-κB antagonist, pyrolidine dithiocarbamate [15]. Promoter analyses in rat VSMC confirm that at least one functional NF-κB binding element in the rat iNOS gene mediates immunostimulant-induced transcription [16].

NF-κB-regulated genes additionally include those which encode cytokines, cytokine-receptors, cell adhesion molecules, major histocompatibility complex proteins and enzymes involved in the synthesis and metabolism of inflammatory mediators (e.g., O_2^-). Transcriptional upregulation of this battery of genes constitutes a phenotypic switch that might be propagated to other cells in a feed-forward manner, where it not for the initiation of anti-inflammatory mechanisms that terminate transcription. One important anti-inflammatory mediator may be NO itself. Indeed, it has been reported that NO inhibits NF-κB activation in vascular cells, including human endothelial cells [17] and rodent VSMC [18]. Suppression of NF-κB activation by NO has been attributed to induction and/or stabilization of IκB-α; this may result from NO-mediated inhibition of IκB-α phosphorylation [15]. Paradoxically, NO has also been reported to trigger NF-κB activation in various situations [19,20]. Explanations for these apparently conflicting actions of NO—anti-inflammatory and pro-inflammatory—include differences in rate, source or duration of cell exposure to NO, differences in the time before measuring NF-κB activity, and cell-specific differences in response to NO. An alternative explanation for the apparent opposing actions of NO is offered by an appreciation that NO can be diverted to reaction products with bioactivities that differ from NO itself. As noted above, co-synthesis of O_2^- in the inflammatory environment effectively channels NO to production of $ONOO^-$. In fact, $ONOO^-$ has been reported to increase iNOS through NF-κB in vascular endothelial cells [21]. Accordingly, we hypothesized that reported NF-κB suppressing and potentiating activities of NO may be reconciled by fundamental differences in actions of NO and $ONOO^-$. The present study compares the effects of NO with $ONOO^-$ on NF-κB activation and iNOS gene expression in VSMC.

2. Methods

2.1. Materials

Recombinant rat interferon-γ, TNFα, and IL-1β were obtained from Genzyme (Cambridge, MA, USA). NOR3 ((±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide), SNAP (S-nitroso-*N*-acetyl-DL-penicillamine), SIN-1 (3-(4-morpholinyl)-sydnonimine hydrochloride), and $ONOO^-$ solutions were purchased from Dojin (Kumamoto, Japan). Bacterial lipopolysaccharide (LPS: *Escherichia coli*, serotype No. 0127:B8), pyrrolidine dithiocarbamate (PDTC), *N*-acetylcysteine (NAC), and 8-bromoguanosine 3′:5′-cyclic monophosphate (8-bromo-cGMP) were obtained from Sigma (St. Louis, MO, USA). Cu/Zn-superoxide dismutase (SOD) was purchased from Roche diagnostic (Tokyo, Japan). Bacteria expressing GST-IκBα (1–55) was kindly provided by Dr. Hideaki Kamata (Himeji Institute of Technology, Japan).

2.2. Cell culture and nitrite assay

VSMC were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats, as previously described [22]. Nitrite accumulation, an indicator of NO synthesis, was measured in the cell culture medium of confluent VSMC, as previously described [14].

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985).

2.3. NF-κB activation

To study NF-κB activation, VSMC were stably transfected with a cis-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFκB-Luc: Stratagene, La Jolla, CA, USA) as previously described [23]. The endothelial cells (YPEN-1 cell line from prostata endothelium, ATCC) were also stably transfected with the same plasmid. Several clones were selected for analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).

For immunohistochemical localization of NF-κB, rat VSMC were grown in wells on glass chamber slides (Nalge Nunc Int., Roskilde, Denmark). Cells were treated with LPS in the absence or presence of NOR3 for 2 h, fixed with 4% formaldehyde/[phosphate-buffered saline (PBS)] for 20 min

at room temperature, and then treated with triton X-100 (0.2% in PBS) for 5 min. Chamber slides were incubated with 1% bovine serum albumin in PBS for 10 min, followed by 30-min exposure to a 1:40 dilution of a rabbit polyclonal antibody that specifically recognizes the p50 subunit of NF-kB (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This p50 antibody did not cross-react with NF-κB subunits p105, p52, or p100 under the conditions employed. Primary antibody was visualized by fluorescence microscopy (Olympus AX 80) after a 30-min incubation with a 1:100 dilution of green-fluorescent Alexa 488 conjugate (goat anti-rabbit IgG, Molecular Probes, Eugene, OR, USA) and washing in PBS.

Electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts, prepared from VSMC that were either untreated or treated with LPS in the absence or presence of NOR3 for 2 h prior to harvesting nuclei. Nuclear extracts were prepared according to the method of Schreiber et al. [24]. EMSA utilized a ^{32}P-labeled double-strand oligonucleotide, containing the NF-κB/c-Rel consensus binding sequence AGTTGAGGGGACTTTCCCAGGC (Promega Biotech, WI, USA). Nuclear proteins were incubated with the labeled oligonucleotide for 30 min and then subjected to polyacrylamide gel electrophoresis and autoradiography.

2.4. iNOS promoter analysis

iNOS promoter activity was studied as previously described [23], using rat VSMC stably transfected with a construct containing a 1.7-kb fragment of the mouse iNOS promoter cloned in front of a reporter gene that encodes the secreted form of human placental alkaline phosphatase (SEAP). SEAP activity in the cell culture medium was measured by a sensitive chemiluminescence assay (Phospha-Light, TROPIX, Bedford, MA, USA).

2.5. Analysis of iNOS, MCP-1, and IκB-α mRNA expression

Standard Northern blotting was used to investigate the mRNA expression for iNOS, MCP-1, and IκB-α, as previously described [23]. After probing for iNOS, MCP-1, or IκB-α expression, filters were stripped and reprobed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Expression of iNOS mRNA was also analyzed by reverse transcription-polymerase chain reaction (RT-PCR) as previously described [25]. IκB-α cDNA was synthesized by amplifying cDNA from VSMC using pri-

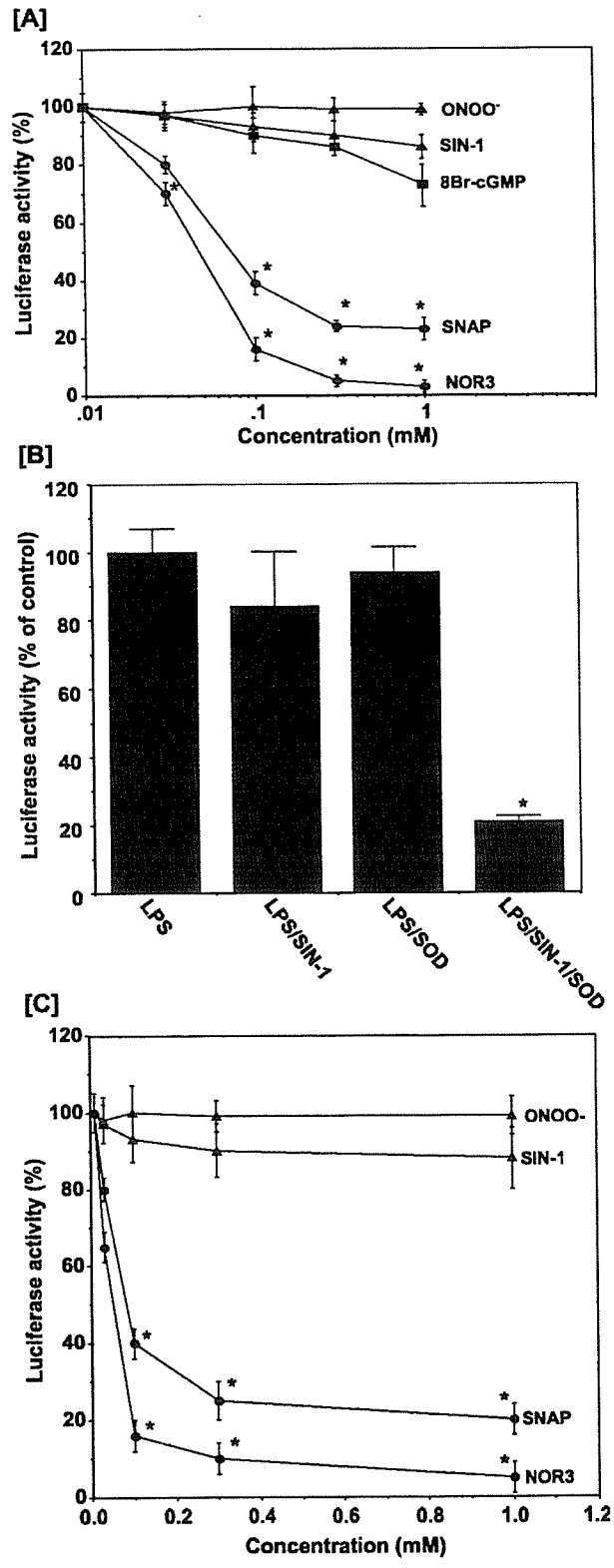

Fig. 1. Effect of NOR3, SNAP, SIN-1, $ONOO^-$, and 8-bromo-cAMP on NF-κB-dependent transcriptional activity/luciferase reporter expression in stably transfected rat vascular smooth muscle cells. (A) Cells were treated with LPS (30 μg/ml) in the presence of indicated concentrations of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP for 3 h. (B) Cells were treated with LPS (30 μg/ml) in the presence of SIN-1 (1 mM) or SOD (5000 U/ml) or both for 3 h. After treatment, luciferase activity in the cells was measured. (C) Using endothelial cells (YPEN-1 cell line from prostata endothelium, ATCC), the effect on NF-κB activation was evaluated. Endothelial cells were treated with LPS (1 μg/ml) in the presence of indicated concentrations of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP for 3 h. Values are means ± S.E.M. ($n = 3$). *$P < 0.05$ versus LPS in the absence of each agent.

mers derived from the published cDNA sequence of murine IκB-α [26]. Sequencing of the PCR product (125 bp) showed 96% identical to the corresponding murine IκB-α cDNA sequence.

2.6. Western blot analysis

VSMC treated with LPS in the presence of NOR3 or $ONOO^-$ for various intervals were lysed using cell lysis buffer (Cell Signaling, Beverly, MA, USA) with 1 mM PMSF. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added at a final concentration of 1%, and each sample was denatured by boiling for 3 min. Samples containing 15 μg of protein were resolved by electrophoresis on 12% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) incubated with Phospho-IκB-α antibody and IκB-α antibody (1:1000, Cell Signaling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG horseradish peroxidase (1:20,000) and the ECL Plus system (Amersham, Buckinghamshire, UK).

2.7. IKK kinase assay

The kinase activity of IκB kinase (IKK) was analyzed by immune complex kinase assay using a substrate, GST-IκBα (1–55), as described previously [27]. Briefly, the cells were solubilized in ice-cold buffer, and then centrifuged at 15,000 × *g* for 20 min. IKKα and IKKβ were recovered from the lysates by immunoprecipitation, and then the immune complexes were incubated with 20 μl reaction buffer containing 20 mM HEPES/NaOH, pH 7.4, 10 mM $MgCl_2$, 50 mM NaCl, 100 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM DTT, 100 μM ATP, 0.1 μCi [γ-^{32}P]ATP, 10 μg GST-IκBα (1–55) at 30 °C for 20 min. After SDS-polyacrylamide gel electrophoresis (PAGE), the phosphorylation of GST- IκBα was estimated by Imaging plate (Fuji Film).

2.8. Statistical analysis

Comparisons between group means were performed by two-way ANOVA and Tukey post hoc analysis test with InStat software (GraphPad Software). Student's unpaired *t*-test was used for comparisons between two treatment groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. NF-κB-dependent transcriptional activation

LPS elicits NF-κB activation in VSMC, thereby stimulating transcription of NF-κB-induced genes. Studies were performed to assess the effect on LPS-induced NF-κB activation of NO-releasing agents (NOR3 and SNAP), an $ONOO^-$ donor (SIN-1), and pure $ONOO^-$. As shown in Fig. 1A, NOR3 and SNAP each caused a dose-dependent and near-complete suppression of NF-κB activation; half-maximal inhibitory concentrations were 50 and 80 μM, respectively. TNFα- and IL-1β also markedly stimulated

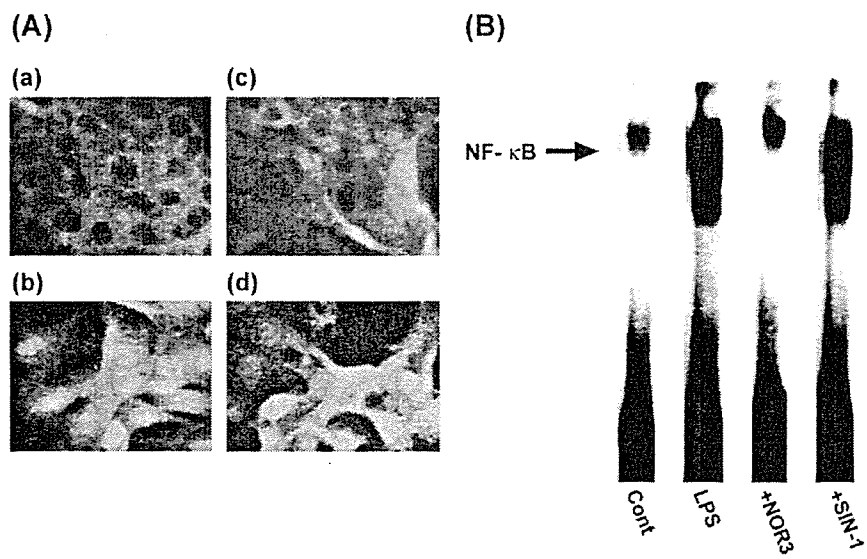

Fig. 2. Effect of NOR3 and SIN-1 on NF-κB nuclear translocation. VSMC were left untreated or treated with LPS (30 μg/ml), alone, or in the presence of NOR3 (500 μM) or SIN-1 (500 μM) for 2 h. (A) Cells were fixed and subjected to immunohistochemical staining for nuclear translocation of NF-κB. (a) Control; (b) LPS; (c) LPS + NOR3; (d) LPS + SIN-1. (B) Cells were treated as in (A), and extracted nuclear protein (10 μg) was subjected to EMSA using a double-stranded oligonucleotide probe for NF-κB binding.

NF-κB activation in VSMC and the NO-donor NOR3 (1 mM) abolished these actions (data not shown). In contrast, no significant inhibition of LPS-induced NF-κB activation was observed when $ONOO^-$ was administered directly or indirectly, as a product of the reaction of SIN-1-derived NO and O_2^-. Notably, when SIN-1 (1 mM) was converted from an $ONOO^-$ donor to an NO donor, by addition of 5000 U/ml SOD, LPS-induced NF-κB activity was potently suppressed (Fig. 1B). Neither SOD on its own (Fig. 1B, 5000 U/ml), nor a cell-permeant analog of cGMP, 8-bromo-cGMP (Fig. 1A), inhibited LPS-induced NF-κB activation. Together, these findings indicate that NO acts by a cGMP-independent mechanism to suppress immunostimulant-induced NF-κB activation and this action is not a property shared with $ONOO^-$.

Using another cell type (endothelial cells), the effect on LPS-induced NF-κB activation of NOR3, SNAP, SIN-1, and pure $ONOO^-$ was examined. As shown in Fig. 1C, NOR3 and SNAP caused a dose-dependent suppression of NF-κB activation in endothelial cells, while SIN-1 and pure $ONOO^-$ had little effect.

3.2. NF-κB nuclear translocation

To examine whether NO prevents nuclear translocation of active NF-κB in LPS treated VSMC, immunohistochemical staining with an anti-p50 antibody was performed. Untreated VSMC displayed a diffuse cytosolic distribution of immunoreactive p50. Following 2-h exposure of rat VSMC to LPS (30 μg/ml), a dense nuclear accumulation of immunoreactive p50 was conspicuous. Notably, the ability of LPS to induce nuclear translocation of NF-κB p50 was abolished by pretreatment of VSMC with NOR3 (500 μM), but unaffected by SIN-1 (500 μM) treatment (Fig. 2A).

EMSA confirmed significant activation of NF-κB by LPS in VSMC. As shown in Fig. 2B, nuclear extracts from LPS-treated cells caused a distinct shift in electrophoretic mobility of a radiolabeled double-stranded oligonucleotide binding probe that recognizes NF-κB; this shifted band was absent from nuclei of untreated cells. This LPS-induced nuclear translocation of active NF-κB was abolished in VSMC that had been pretreated with NOR3 (500 μM), as evidenced by loss of the shifted-band (Fig. 2B). In contrast

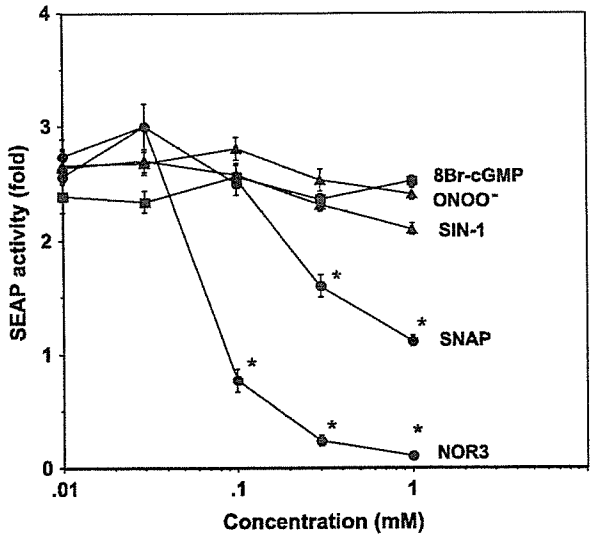

Fig. 3. Effect of NOR3, SNAP, SIN-1, $ONOO^-$, and 8-bromo-cAMP on iNOS promoter/SEAP reporter expression in stably transfected rat VSMC. Cells were treated with LPS (30 μg/ml) alone, or in the presence of indicated concentrations of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP for 24 h. After this time, SEAP activity was quantified in the cell culture medium. Values are means ± S.E.M. ($n=3$). *$P<0.05$ versus LPS alone.

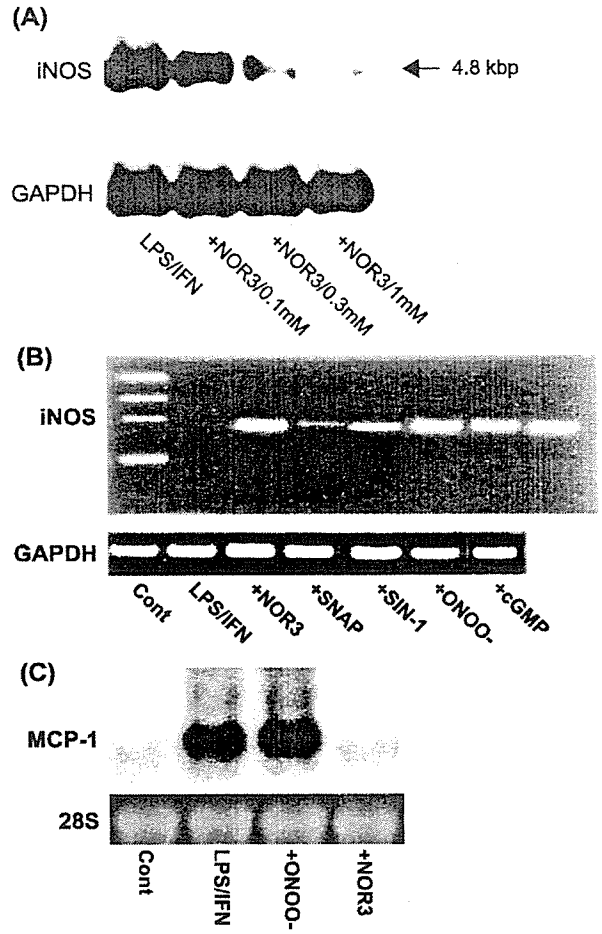

Fig. 4. Effect of NOR3, SNAP, SIN-1, $ONOO^-$, or 8-bromo-cAMP on LPS/IFN-induced increase in iNOS mRNA abundance. Rat VSMC were treated with LPS (30 μg/ml) plus IFN (100 U/ml) in the presence of indicated concentrations of NOR3 (panel A), or 1 mM concentrations of NOR3, SNAP, SIN-1, $OONO^-$, or 8-bromo-cAMP (panel B) for 8 h. For MCP-1 gene expression, VSMC were treated with LPS/IFN in the presence of NOR3 (1 mM) or $ONOO^-$ (1 mM) for 6 h (panel C). Total RNA was isolated and analyzed by northern blot hybridization with an iNOS-specific probe (A) and with MCP-1-specific probe (C), or RT-PCR using iNOS-specific primers (B).

to the complete suppression of NF-κB activation observed with an NO-donor, an $ONOO^-$ donor SIN-1 (500 mM) was without effects (Fig. 2B).

3.3. iNOS promoter activation

The effects of NOR3, SNAP, SIN-1, $ONOO^-$, and 8-bromo-cGMP on LPS-induced iNOS promoter activation in VSMC were evaluated. LPS potently activated the iNOS promoter, as indicated by a >5-fold increase in SEAP reporter gene activity (Fig. 3). This LPS-induced iNOS promoter activity was inhibited in a dose-dependent manner by NOR3 (IC_{50} = 70 μM) and SNAP (IC_{50} = 175 μM). In contrast, SIN-1, peroxynitrite, and 8-bromo-cGMP had no significant inhibitory effect on iNOS promoter activation by LPS (Fig. 3).

3.4. mRAN expression of iNOS and MCP-1

While iNOS mRNA levels approached the detection-limit by northern blot analysis in unstimulated VSMC, the combination of LPS/IFN provided a strong stimulus for iNOS mRNA expression (Fig. 4A). The LPS/IFN-induced increase in iNOS mRNA level was substantially decreased in cells that had been co-treated with NOR3 (0.1 to 1 mM). Fig. 4B shows the effects of NOR3, SNAP, SIN-1, peroxynitrite, and 8-bromo-cGMP on LPS-induced iNOS mRNA levels, as evaluated by RT-PCR. iNOS mRNA was barely detectable by RT-PCR in unstimulated VSMC, but substantially upregulated by 8 h after treatment with LPS/IFN. The LPS/IFN-induced increase in expression of iNOS mRNA was blocked by NOR3 and diminished by SNAP. In contrast, no substantial effect of SIN-1, $ONOO^-$, or 8-bromo-cGMP could be detected on LPS-induced iNOS mRNA expression.

To study the effects of NO and peroxynitrite on other gene expression, MCP-1 mRNA levels were evaluated in LPS/IFN-stimulated VSMC (Fig. 4C). The LPS/IFN-induced increase in MCP-1 mRNA levels was substantially decreased by NOR3, while the levels of MCP-1 mRN levels were not changed in response to $ONOO^-$.

3.5. Antioxidants inhibit NF-κB and NO synthesis

Pyrrolidine dithiocarbamate (PDTC) and *N*-acetylcysteine (NAC) are antioxidants that have been shown to prevent NF-κB activation in many experimental settings. As shown in Fig. 5A, NAC (1 mmol/l) and PDTC (50 μmol/l) markedly attenuated LPS/IFN-induced NF-κB activity in VSMC (62% and 85%, respectively). NAC and PDTC similarly attenuated LPS/IFN-induced NO synthesis (72% and 90%, respectively; Fig. 5B).

3.6. LPS stimulates IκB phosphorylation by inducing IKK activity, and NOR3 inhibits LPS-induced IκB phosphorylation and IKK activity

We first determined whether LPS-induced NF-κB activation is occurred through phosphorylation/degradation of IκB. To determine whether LPS causes IκB-α phosphorylation in rat VSMCs, Western blot analysis using anti-phospho-Ser32 of IκB-α antibody was performed. LPS induces IκB phosphorylation in 15 min, and the levels of phospho-IκB-α disappeared in 60 min. (Fig. 6A, upper panel). The blot was reprobed with anti-IκB antibody, and the data indicated that the maximum LPS-induced degra-

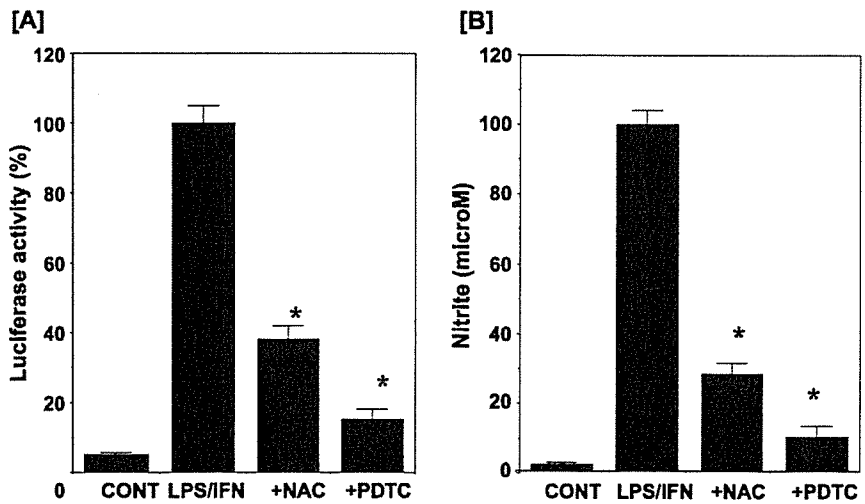

Fig. 5. Effect of NAC or PDTC on nitrite production and NF-κB-dependent transcriptional activity/luciferase reporter expression. Rat VSMC cells were treated with LPS (30 μg/ml) plus IFN (100 U/ml) in the presence of NAC (1 mM) or PDTC (50 μM). (A) Luciferase activity in the cells was measured after a 3 h incubation, and (B) nitrite accumulation in the culture medium was measured after a 24 h. Data are means ± S.E.M. (n = 3). *P < 0.05 versus LPS/IFN in the absence of NAC or PDTC.

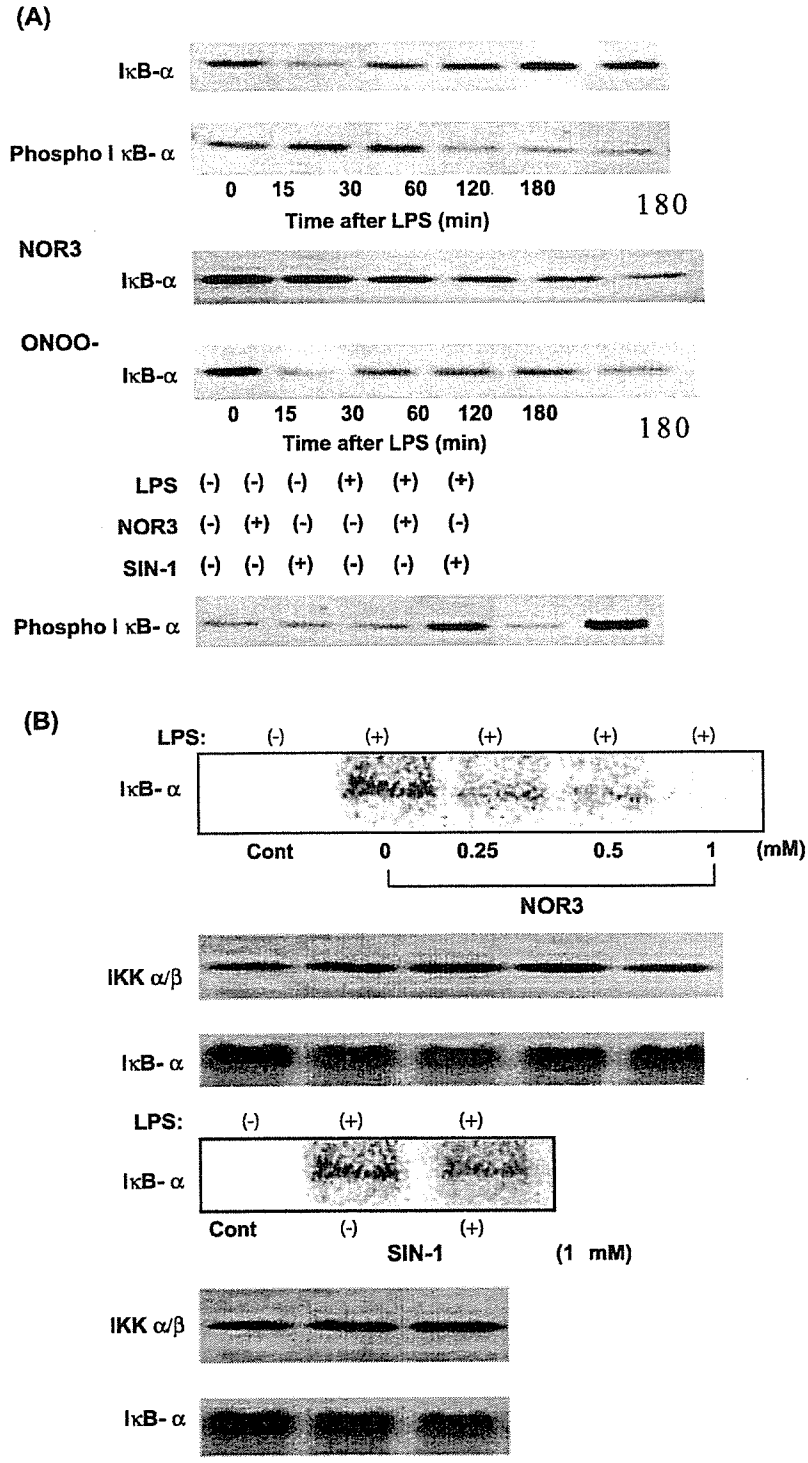

Fig. 6. (A) Effect of NOR3 and SIN-1 on degradation and phosphorylation of I B-α in rat VSMC. Cells were incubated for 45 min with various concentrations of NOR3 (1 mM) or SIN-1 (1 mM), followed by LPS (30 μg/ml) for 0–180 min. Cell were lysed and subjected to Western blot analysis using anti-I B-α antibody and anti-phospho-I B-α. (B) Effect of NOR3 and SIN-1 on IKK activity in rat VSMC. Cells were incubated for 45 min with various concentrations of NOR3 (0.25–1 mM) or SIN-1 (1 mM), followed by LPS (30 μg/ml) for 15 min. Cells were lysed and immunoprecipitated with anti-IKKα/β antibody and used for kinase assay using recombinant I B-α as substrate. Note that equal intensities of IKKα/β- and I B-α-specific bands are obtained.

dation was observed within 30 min. After that, IκB synthesis was reactivated possibly by NF-κB in 120 min (upper panel). Next, the effect of NOR3 or ONOO- on LPS-induced IκB-α degradation was determined (middle panel). NOR3 completely blocked this IκB-α degradation. SIN-1 had no effect on LPS-induced IκB-α degradation (middle panel). Lower panel shows the effect of NOR3 and SIN-1 on the phosphorylation of IκB-α. Addition of LPS resulted in a rapid (within 15 min) appearance of phosphorylated IκB-α. NOR3 (10^{-3} mol/l) prevented the LPS-stimulated increase in phosphorylated IκB-α. While SIN-1 had no effect on the LPS-stimulated phosphorylation of IκB-α (lower panel).

The radiolabeled, phosphorylated IκB-α-specific band is detected in LPS-treated cells while it was undetectable in the untreated cells, demonstrating that LPS induces the IKK activity (Fig. 6B) This IKK activity was dose-dependently decreased by treatment of the cells with NOR3 (upper panel). However, SIN-1 had no effect on LPS-induced IKK activity (lower panel). The remaining half of the immunoprecipitated samples were analyzed by Western blot analysis using anti-IKKα/β antibody, showing the identical level of expression of IKK, suggesting that IKK is expressed in these cells. The identical amount of IκB was detected when the equal volume of kinase reaction mixture was loaded into SDS-PAGE and analyzed by Western blot using anti-IκB antibody (Fig. 6B).

3.7. IκB-α mRNA expression

IκB-α mRNA expression was expressed at a relatively low level in unstimulated VSMC, as measured by northern blot analysis. Treatment of cells with LPS for 2 h substantially increased levels of IκB-α mRNA. NOR3 (1 to 1000 μM) alone neither increased the abundance of IκB-α mRNA, nor did it (100 μM) influence the extent of upregulation by LPS (data not shown).

4. Discussion

A wealth of literature supports the notion that ONOO is a key mediator of cytotoxicity arising from NO synthesis. Herein we reveal a fundamental and potentially critical distinction between NO and $ONOO^-$: while NO acts to inhibit NF-κB activation, $ONOO^-$ allows for sustained activation of NF-κB. This conclusion in VSMC is uniformly supported by results from several lines of investigation: (a) NF-κB-driven reporter gene expression, (b) EMSA studies of NF-κB nuclear translocation, and (c) immunocytochemical analysis of NF-κB p50 subcellular localization. Given that iNOS gene transcription itself requires NF-κB for upregulation by immunostimulants [7,8,15,16], it is also revealing that NO, but not $ONOO^-$, suppressed immunostimulant-evoked iNOS promoter activity and mRNA expression. The ability of NO to suppress NF-κB activation extends from iNOS to the numerous NF-κB-dependent gene products that contribute to the inflammatory response. That endogenously produced NO can indeed function as an anti-inflammatory molecule in biological systems is supported by reports that NOS inhibitors potentiate cytokine-induced expression of NF-κB-regulated genes in endothelial cells [28], macrophages [29] and in tissues from animals treated with immunostimulants [30] or exposed to ischemia–reperfusion injury [31].

Is the inhibition of NF-κB that we observe with NO-donors actually mediated by NO? Amperometric detection indicates that all NO donors tested—NOR3, SNAP and SIN-1 (in the presence of SOD)—release NO in physiological buffers at 37 °C with a $T1/2 < 2$ h [32,33]. Given the diverse structure of these compounds, it is unlikely that their shared ability to prevent NF-κB activation resides in either the parent or product molecules. We cannot however exclude the possibility that a NO-derived species, other than $ONOO^-$, (e.g., a nitrosothiol) is responsible or contributes to NF-κB inhibition.

High reactivity would predict that all added $ONOO^-$ is consumed within seconds of addition to cells. Accordingly, the failure of pure $ONOO^-$ to elicit effects on NF-κB activation or iNOS gene expression could conceivably be due to a more limited exposure of cells to $ONOO^-$, compared with NO from donor compounds. Studies with SIN-1 argue against this view. SIN-1 has the unique property of generating $OONO^-$ by releasing equimolar quantities of O_2^- and NO, essentially in a simultaneous manner. Although SIN-1 delivers a continuous flux of $ONOO^-$ to VSMC, no effect on LPS-induced NF-κB activation and iNOS gene expression was observed. The concentration of SIN-1 used in our study (1 mM) was reported to produce $ONOO^-$ under the conditions employed, but no detectable NO [34]. However, release of NO by SIN-1 can be observed to occur as an increasing function of added SOD, with ~5000 units achieving complete scavenging of O_2^- and maximal stimulation of NO production [35]. Our data show that only when SIN-1 was transformed into a pure NO donor (upon addition of 5000 units of SOD) did it substantially inhibit LPS-induced NF-κB activation. Thus, SIN-1 generated a sufficient flux of $ONOO^-$, under the experimental conditions used, that diversion to NO resulted in potent suppression of NF-κB activation. Accordingly, findings with SIN-1 provide strong support for the view that $ONOO^-$ supports, rather than suppresses, NF-κB activation.

A cGMP-independent effect of NO is suggested by our observation that a cell-permeable cGMP analogue failed to elicit a detectable effect on either NF-κB activation or iNOS gene expression. We confirmed that NO inhibits iNOS gene expression by directly blocking phosphorylation and subsequent degradation of IκB-α [18]. We further showed that LPS stimulates IκB-α phosphorylation by inducing IKK activity, and NO inhibits LPS-induced IκB-α phosphorylation and IKK activity. These data suggested that NO suppressed the

LPS-induced NF-κB activation at a step prior to the IκB phosphorylation. One line of evidence suggests that NO inhibits NF-κB activation by modulating IκB. NO was reported to induce IκB-α expression and nuclear translocation in human endothelial cells [17,35]. While we found that LPS substantially upregulated IκB-α mRNA expression, perhaps contributing to the development of *tolerance* to LPS effects [36], NO failed to modulate IκB-α mRNA levels. Evidence also supports the view that NO can nitrosylate a redox-active cysteine residue in NF-κB that attenuates binding to DNA in vitro [37] and in cells [38]. Another potential mechanism for the NF-κB-suppressant action of NO is direct scavenging of O_2^- (or other pro-oxidants) that might otherwise activate NF-κB. This is in accord with our observation that the radical scavengers NAC and PDTC each block LPS-induced NF-κB activation and NO synthesis in VSMC.

Pioneering work of Beckman et al. [39] first recognized $ONOO^-$ to be a highly reactive molecule whose synthesis hung in balance with physiological NO. Tipping this balance toward $ONOO^-$, by enhanced production of O_2^-, has served to explain many of the cytotoxic actions of NO. The present findings offer a novel and potentially important extension to the earlier yin/yang view: NO inhibits while $ONOO^-$ supports immunostimulant-induced NF-κB activation and iNOS gene expression in VSMC. In this context, we ascribe the cytotoxicity of $ONOO^-$ to the loss of a benevolent action of NO. Appreciation that this chemical switch toggles the inflammatory response recommends a therapeutic goal be upregulating the NO/O_2^- balance to achieve vasoprotection and combat inflammatory disease.

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ARTICLE

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Angiotensin-II-induced oxidative stress elicits hypoadiponectinaemia in rats

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Abstract *Aims/hypothesis:* Hypertension, endothelial dysfunction and insulin resistance are associated conditions that share oxidative stress and vascular inflammation as common features. Adiponectin is an abundant plasma adipokine that plays a physiological role in modulating lipid metabolism and exerts a potent anti-inflammatory activity. We hypothesised that adiponectin levels decrease in response to oxidative stress and that this may promote the development of hypertension, endothelial dysfunction and insulin resistance. *Methods:* Rats were infused with angiotensin II (AngII) or its vehicle, either alone or in combination with tempol (4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl), a membrane-permeable metal-independent superoxide dismutase mimetic, or tetrahydrobiopterin (BH4), one of the most potent naturally occurring reducing agents and an essential cofactor for nitric oxide synthase activity. Heart rate, systolic blood pressure, body weight and serum levels of adiponectin were measured on day 7 of treatment, and then the animals were killed. Vessel tone and superoxide production were measured ex vivo in thoracic vascular rings. The expression of adiponectin mRNA in adipose tissue was assessed by Northern blotting, and in 3T3-L1 adipocytes exposed to H_2O_2 by real-time PCR. The expression of NAD(P)H oxidase subunit mRNAs in the rats was assessed by RT-PCR and real-time PCR. *Results:* Hypertension and endothelial dysfunction were induced in rats by infusion of AngII and reversed by administration of tempol. Plasma concentrations of adiponectin and adipose tissue levels of adiponectin mRNA were decreased in AngII-infused rats, and this effect was prevented by cotreatment with tempol or BH4. The production of superoxide anions (O_2^-) was significantly increased in the aortae of AngII-treated rats, and this increase was prevented by the administration of tempol or BH4. Levels of mRNAs that encode NAD(P)H oxidase components, including p22phox, gp91phox, p47phox and Rac1, were similarly increased in adipose tissue, aortae and hearts of AngII-infused rats. Cotreatment of rats with tempol or BH4 reversed AngII-induced increases in NAD(P)H oxidase subunit mRNAs. Fully differentiated 3T3-L1 adipocytes, also exhibited diminished adiponectin mRNA levels when exposed to low concentrations of H_2O_2. *Conclusions/interpretation:* Our results demonstrate that AngII-induced oxidative stress and endothelial dysfunction are accompanied by a decrease in adiponectin gene expression. Since antioxidants were observed to prevent the actions of AngII, and H_2O_2 on its own suppressed adiponectin expression, we conclude that adiponectin gene expression is negatively modulated by oxidative stress. Plasma adiponectin levels may provide a useful indicator of oxidative stress in vivo, and suppressed levels may contribute to the proinflammatory and metabolic derangements associated with type 2 diabetes, coronary artery disease and the metabolic syndrome.

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Abbreviations AngII: angiotensin II · BH4: tetrahydrobiopterin · AT1: angiotensin type 1 · GAPDH: glyceraldehyde-3-phosphate dehydrogenase · NO: nitric oxide · PSS: physiological salt solution · ROS: reactive oxygen species

Introduction

Adiponectin is an important adipocytokine that is secreted by adipocytes and circulates at relatively high levels in the

bloodstream. Adiponectin has potent anti-inflammatory and atheroprotective effects on vascular tissue, and has an insulin-sensitising effect on tissues involved in glucose and lipid metabolism. Adiponectin levels are decreased in patients with, and animal models of, obesity, diabetes and coronary artery disease [1–5]. This observation, combined with the fact that adiponectin has a number of vascular protective effects [6–10], suggests that the decreased plasma adiponectin levels associated with obesity and diabetes may contribute to the development of vascular disease in these patients. However, the mechanism by which adiponectin levels are decreased remains unknown. Vascular endothelial dysfunction plays a pivotal role in the pathogenesis of atherosclerosis and increases the risk of future cardiovascular events [11, 12]. Adiponectin stimulates nitric oxide (NO) production in vascular endothelial cells [13]. In addition, hypoadiponectinaemia has been linked to endothelial dysfunction in humans [14, 15]. Thus, the observed relationship between insulin resistance and vascular endothelial cell dysfunction may be related to reduced levels of adiponectin.

Angiotensin II (AngII) exerts multiple effects on the cardiovascular system, including elevation of blood pressure, vascular endothelial dysfunction, and cardiovascular hypertrophy. AngII-induced cardiovascular alterations may be the result of free radical production [16]. Through its type 1 (AT1) receptor, AngII stimulates the overexpression of cytosolic proteins involved in the activation of NAD(P)H oxidase within vascular endothelial cells, smooth muscle cells and leucocytes [17, 18], which favours the production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl radicals.

AngII also induces insulin resistance via oxidative stress [19]. Recent clinical trials suggest that blockade of the renin–angiotensin system, either by inhibition of angiotensin-converting enzyme (ACE) [20, 21] or blockade of the AT1 receptor [22], may substantially reduce the risk of developing type 2 diabetes, although the mechanism responsible for this effect has yet to be elucidated. Given that AngII inhibits the adipogenic differentiation of human adipocytes via the AT1 receptor [23], and that the expression of AngII-forming enzymes in adipose tissue is inversely correlated with insulin sensitivity [24], it has been suggested that blockade of the renin–angiotensin system might prevent the development of diabetes by promoting adipocyte differentiation. The increased production of AngII by large, insulin-resistant adipocytes inhibits the recruitment of preadipocytes, resulting in the increased storage of lipid in muscle and other tissue, thereby decreasing insulin sensitivity.

In the present study we examined the influence of AngII infusion on adiponectin expression in rats. Based on the finding that AngII infusion elicits a significant and profound decrease in circulating adiponectin, we investigated the possibility that oxidative stress might underlie AngII-induced hypoadiponectinaemia by examining the effect of tempol (4-hydroxy-2,2,6,6,-tetramethylpiperidin-1-oxyl) [25, 26], a membrane-permeable superoxidase dismutase mimetic, and tetrahydrobiopterin (BH4) [27, 28], one of the most potent naturally occurring reducing agents and an essential cofactor for enzymatic NO synthase activity.

Materials and methods

Animals and experimental protocol The present experiment was reviewed and approved by the Committee on Ethics of Animal Experiments and conducted according to the Guidelines for Animal Experiments, Dokkyo University Faculty of Medicine.

Seven-week-old male Sprague–Dawley rats (Tokyo Experimental Animals, Tokyo, Japan) were randomly divided into six experimental groups of eight rats. The rats were infused with AngII or its vehicle (distilled water), either alone (AngII and control groups, respectively) or in combination with tempol (AngII-tempol and tempol groups, respectively) or BH4 (AngII-BH4 and BH4 groups, respectively). AngII (Sigma, St Louis, MO, USA) was infused subcutaneously using an osmotic pump (model 2002; Alza Corporation, Palo Alto, CA, USA) at a dose of 300 $ng \cdot kg^{-1} \cdot min^{-1}$ for 7 days. Tempol (Wako Pure Chemical Industries, Tokyo, Japan) and BH4 (sapropterin; a generous gift from Daiichi Suntory Pharma, Tokyo, Japan) were administered in the drinking water (2 and 0.2 mg/ml, respectively), 24 h before and during the 7-day period of AngII infusion.

Vessel collection and adipose tissue preparation On day 7 of treatment, the heart rate and systolic blood pressure of the rats were measured using the tail cuff method. The rats were anaesthetised with intraperitoneally administered pentobarbital, and the chest was opened. With the heart still beating, heparin (150 IU) was given via intracardiac injection. The thoracic aorta was removed en bloc and placed in cold Krebs–Henseleit solution. Extravascular tissue was rapidly removed, and the vessel lumen was flushed with solution. Some of the aortas were cut into three 5-mm ring segments for use in studies of vasoreactivity and superoxide anion production.

Adipose tissue was also obtained from the peritoneal fat pad in order to measure the levels of mRNAs encoding adiponectin and NADPH oxidase-related proteins.

Measurement of adiponectin levels in serum Serum concentrations of adiponectin were determined by ELISA using a kit for the measurement of rat/mouse adiponectin (Otsuka Pharmaceuticals, Tokyo, Japan).

Organ chamber experiments Organ chamber experiments were performed as previously described [29]. Animals were anaesthetised with pentobarbital and then exsanguinated. The thoracic aortas were carefully dissected, and all perivascular tissue removed under a microscope in a physiological salt solution (PSS) of the following composition (in mmol/l): NaCl 121, KCl 4.7, $NaHCO_3$ 24.7, $MgSO_4$ 12.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, glucose 5.8; aerated with 95% O_2, 5% CO_2. In some experiments, the endothelium was denuded by gentle rubbing of the luminal surface with an appropriate silk. The rings of each thoracic aorta (5 mm in length) were mounted vertically between two hooks in organ chamber myographs (Medical Supply Company, Tokyo, Japan), which were filled with PSS and kept at

37°C. Isometric tension was measured with force transducers (Nihon Kohden, Tokyo, Japan). Each preparation was stretched to an optimal length in a stepwise manner, at which point the force induced by 118 mmol/l KCl was maximal and constant. After equilibration for at least 30 min, the rings were precontracted with prostaglandin F_2 (3–10 μmol/l). Once a stable contraction was achieved, the rings were exposed to acetylcholine (10^{-10} to 10^{-5} mol/l) to evaluate endothelial vasodilator function. Endothelium-independent relaxation in response to sodium nitroprusside (10^{-11} to 10^{-6} mol/l) was examined in endothelium-denuded rings.

Measurement of vascular superoxide anion production Superoxide anion production was measured using lucigenin (bis-*N*-methylacridinium nitrate) chemiluminescence, as previously described [29]. Briefly, the thoracic aortas were carefully dissected, and all perivascular tissue and contaminating blood products were removed in PSS under a microscope, after which the aortas were placed in HEPES-buffered PSS. In a preliminary study we confirmed that no adhesion of inflammatory cells to the endothelium occurred (data not shown). Scintillation vials containing 1 ml HEPES-buffered PSS with 5 μmol/l lucigenin were placed into a scintillation counter (Luminescence Reader BLR 301; Aloka, Tokyo, Japan). To validate our method we used tiron (4,5-dihydroxy-1,3-benzene disulphonic acid; 10 mmol/l), a superoxide scavenger, in all experiments. After dark adaptation, background counts were recorded for 3 min, after which three vascular segments (5 mm in length) from each thoracic aorta were added to each vial. Scintillation counts were then recorded every minute for 10 min and the respective background counts subtracted. The vessels were then dried for determination of dry weight. Lucigenin counts were expressed as counts per minute per milligram of dry weight. The measurements were also performed in the presence of the NAD(P)H oxidase inhibitor apocynin (100 μmol/l), which inhibits the assembly of the components of the enzyme [30, 31].

Measurement of levels of adiponectin and NADPH oxidase mRNAs in adipose tissue Standard Northern blotting was used to investigate the expression of adiponectin mRNA in adipose tissue, as previously described [32]. After probing for adiponectin, filters were stripped and re-probed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Radioactivity on the blots was quantified using an image analyser (BAS2000; Fuji Film, Tokyo, Japan). The expression of p22phox, gp91phox, p47phox, Rac1 and GAPDH mRNAs was analysed by RT-PCR, as previously described [33]. The NAD(P)H oxidase components were quantified by amplification of cDNA using an ABI Prism 7000 real-time thermocycler (Applied Biosystems, Foster City, CA, USA). Copy numbers of the transcripts were obtained from standard curves generated from rat p22phox, gp91phox, p47phox and Rac1 templates [34].

Cell culture and RT-PCR The 3T3-L1 pre-adipocytes (American Type Culture Collection, Manassas, VA, USA) were grown to confluence in DMEM containing 25 mmol/l glucose, as described previously [35]. Forty-eight hours following confluence, the cells were induced to differentiate into adipocytes 48 h after confluence by changing the medium to DMEM supplemented with 10% FCS, 5 μg/ml recombinant human insulin, 0.5 mmol/l isobutylmethylxanthine and 0.25 μmol/l dexamethasone for 48–72 h. The cells were used 9 or 10 days after the induction of differentiation, when more than 90% of the cells exhibited an adipocyte phenotype. The addition of glucose oxidase at concentrations of up to 100 mU/ml (type II from *Aspergillus niger*, 20,000 U/g solid in non-oxygen-saturated conditions; Sigma) to serum-free DMEM supplemented with 0.5% RIA-grade bovine serum albumin was used to generate H_2O_2 [35]. Total RNA was isolated from the cells and reverse transcribed. Adiponectin mRNA was quantified by amplification of cDNA using an ABI Prism 7000 real-time thermocycler. Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan. To examine the cytotoxic effect of glucose oxidase, the cells were incubated (37°C) with MTT (0.4 mg/ml) for a further 60 min after exposure to glucose oxidase. Culture medium was removed by aspiration, and the cells were solubilised in DMSO. The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}. Values were compared with those obtained for the control cells (no glucose oxidase).

Statistical analysis Data are expressed as means±SEM. Differences between two experiments were compared by Student's *t*-tests. Differences between three experiments were determined by two-way ANOVA and Bonferroni's multiple comparison test. A p value of 0.05 was considered statistically significant.

Results

Body weight and haemodynamic parameters The infusion of AngII alone elicited a profound pressor effect during the 7-day treatment period (48.8% increase in systolic blood pressure vs vehicle-infused rats; $p<0.05$) (Table 1). The

Table 1 Body weight and systolic blood pressure of the rats following infusion

	Control group	AngII group	Tempol group	AngII–tempol group	BH4 group	AngII–BH4 group
Body weight (g)	313±12	258±8**	295±10	275±9*	321±12	282±10
Systolic blood pressure (mmHg)	125±8	186±11**	123±11	142±7	125±7	136±12

Data are means±SEM
* $p<0.05$, ** $p<0.01$ vs control group

AngII-induced increase in blood pressure was accompanied by a 17.5% decrease in body weight ($p<0.01$). The unrestricted administration of either tempol (2 mmol/l) or BH4 (0.2 mg/ml), both of which are anti-oxidants, had no significant effect on rat body weight or systolic blood pressure. However, each agent effectively prevented the weight loss and pressor actions of AngII (Table 1).

Angiotensin-II-induced endothelial dysfunction Acetylcholine induced relaxation of aortic rings in an endothelium-dependent manner (Fig. 1). The vasorelaxation of aortic rings from AngII-infused rats was significantly impaired compared with that of rings from vehicle-infused control rats (Fig. 1a). This impairment was characterised by a ≈30% reduction in maximal acetylcholine-induced vasorelaxation and a marked rightward shift in the acetylcholine concentration–response curve. In contrast, AngII did not diminish the maximal vasorelaxant action of sodium nitroprusside, an endothelium-independent (NO-mediated) vasodilator, and caused only a small rightward shift in the concentration–response relationship (Fig. 1b). Since acetylcholine-induced vasorelaxation is mediated by NO in this system, our findings are consistent with the view that AngII promotes vasoconstriction by reducing levels of endothelial-derived NO, rather than diminishing the smooth muscle response to NO. Tempol and BH4 significantly ameliorated AngII-induced endothelial dysfunction (Fig. 1a), and had no effect on the endothelium-independent vasorelaxation induced by sodium nitroprusside (Fig. 1b). Of note, the tempol- and BH4-mediated improvements in endothelial vasodilator function were abolished in the presence of L-NAME (N^G-nitro-L-arginine methyl ester; 100 μmol/l), indicating that tempol and BH4 exert their beneficial effects through the restoration of NO bioactivity (data not shown).

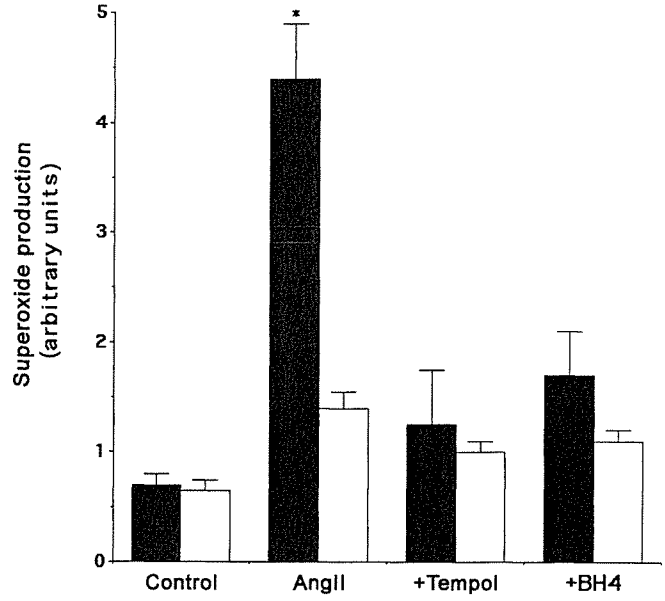

Fig. 2 Superoxide production in thoracic aortic rings in the absence (*closed bars*) and presence (*open bars*) of apocynin. Long-term treatment with tempol or BH4 suppressed the AngII-induced endothelial production of superoxide anions. The AngII-induced increase in superoxide production was acutely and significantly attenuated in the presence of apocynin (100 μmol/l). Results are expressed as means± SEM. Six to eight rings were used to determine the mean values. *$p<0.01$ vs the control value

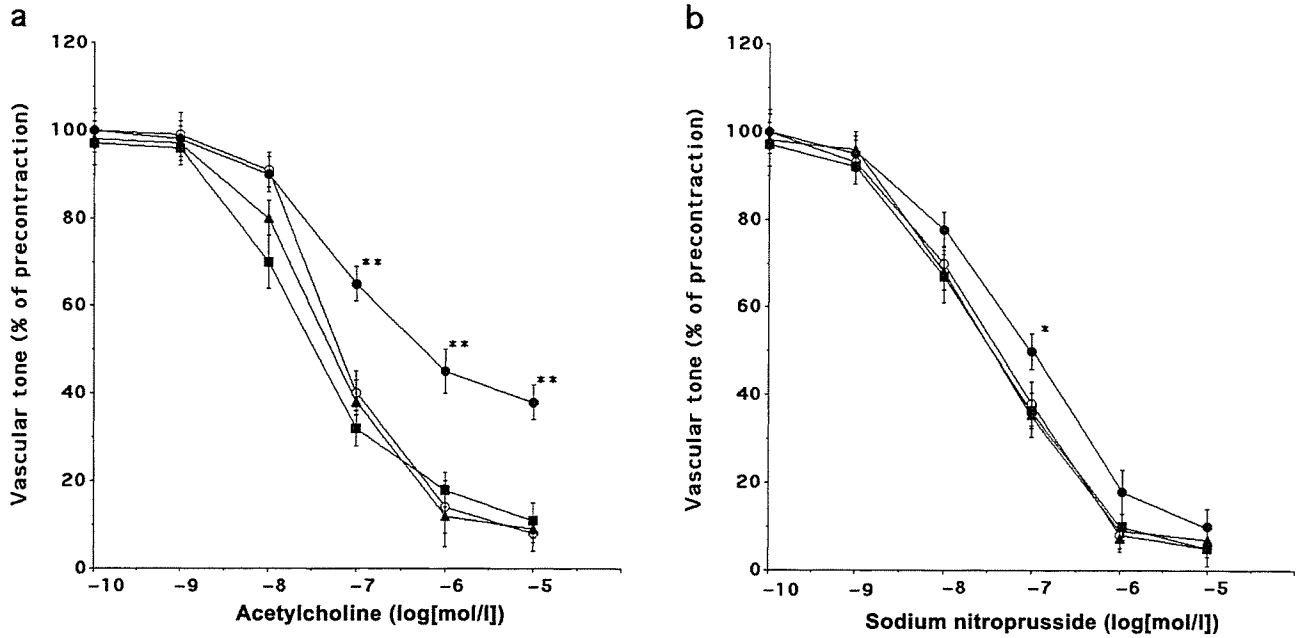

Fig. 1 Endothelium-dependent relaxation in response to acetylcholine (**a**) and endothelium-independent relaxation in response to the NO donor sodium nitroprusside (**b**) in thoracic aortic rings from control animals (*open circles*) and rats treated with AngII, either alone (*closed circles*) or in combination with tempol (*closed triangles*) or BH4 (*closed squares*). The data represent the means±SEM of six to eight vascular rings. *$p<0.05$, **$p<0.01$ vs the control value

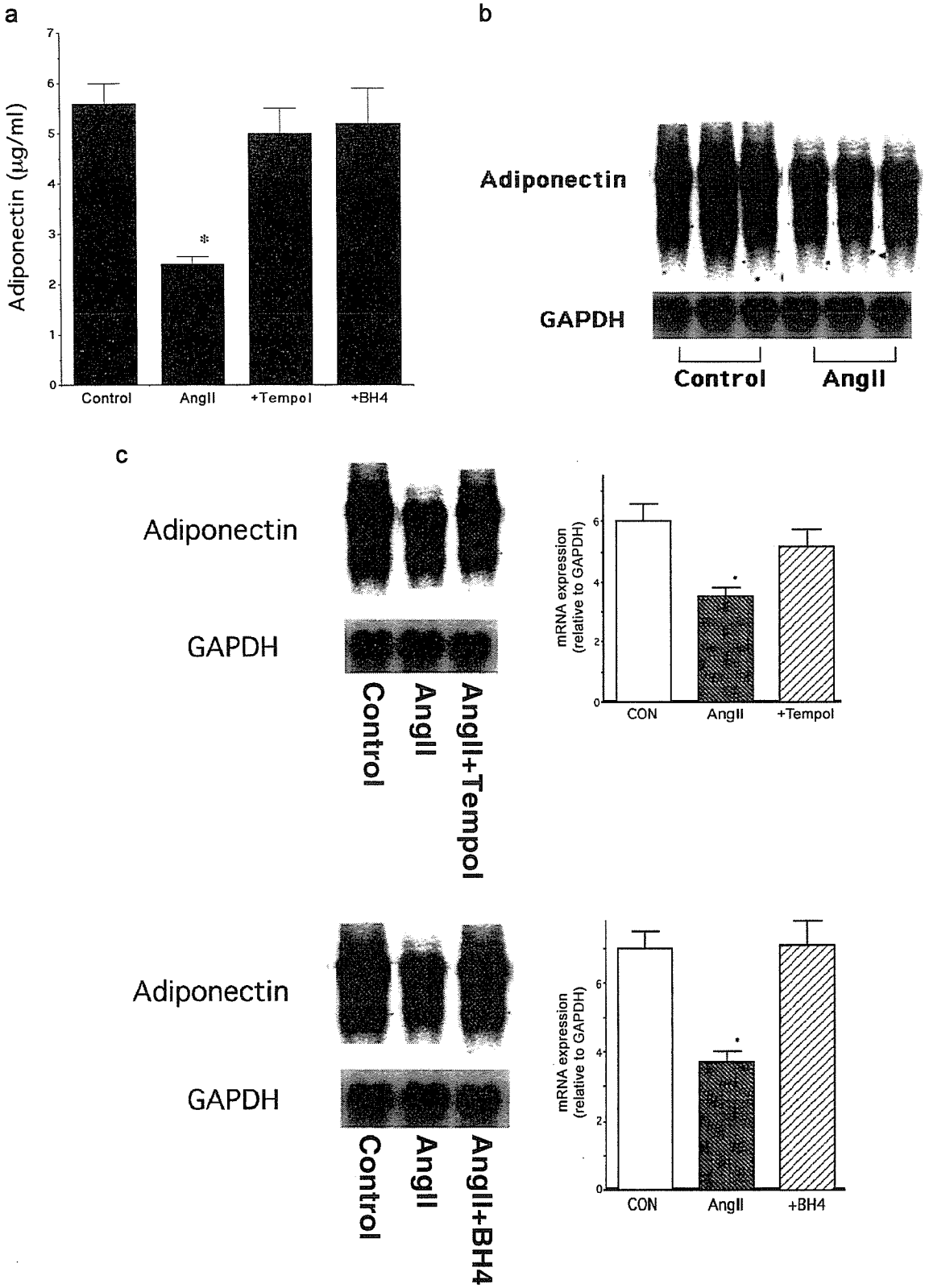

Fig. 3 **a** Plasma adiponectin levels in adipose tissue as determined by ELISA using a kit for the measurement of rat/mouse adiponectin. The results are expressed as means±SEM (n=7). **b**, **c** Adiponectin mRNA levels in adipose tissue as assessed by northern blot analysis. The results are expressed as means±SEM (n=3). *p<0.01 vs the control value

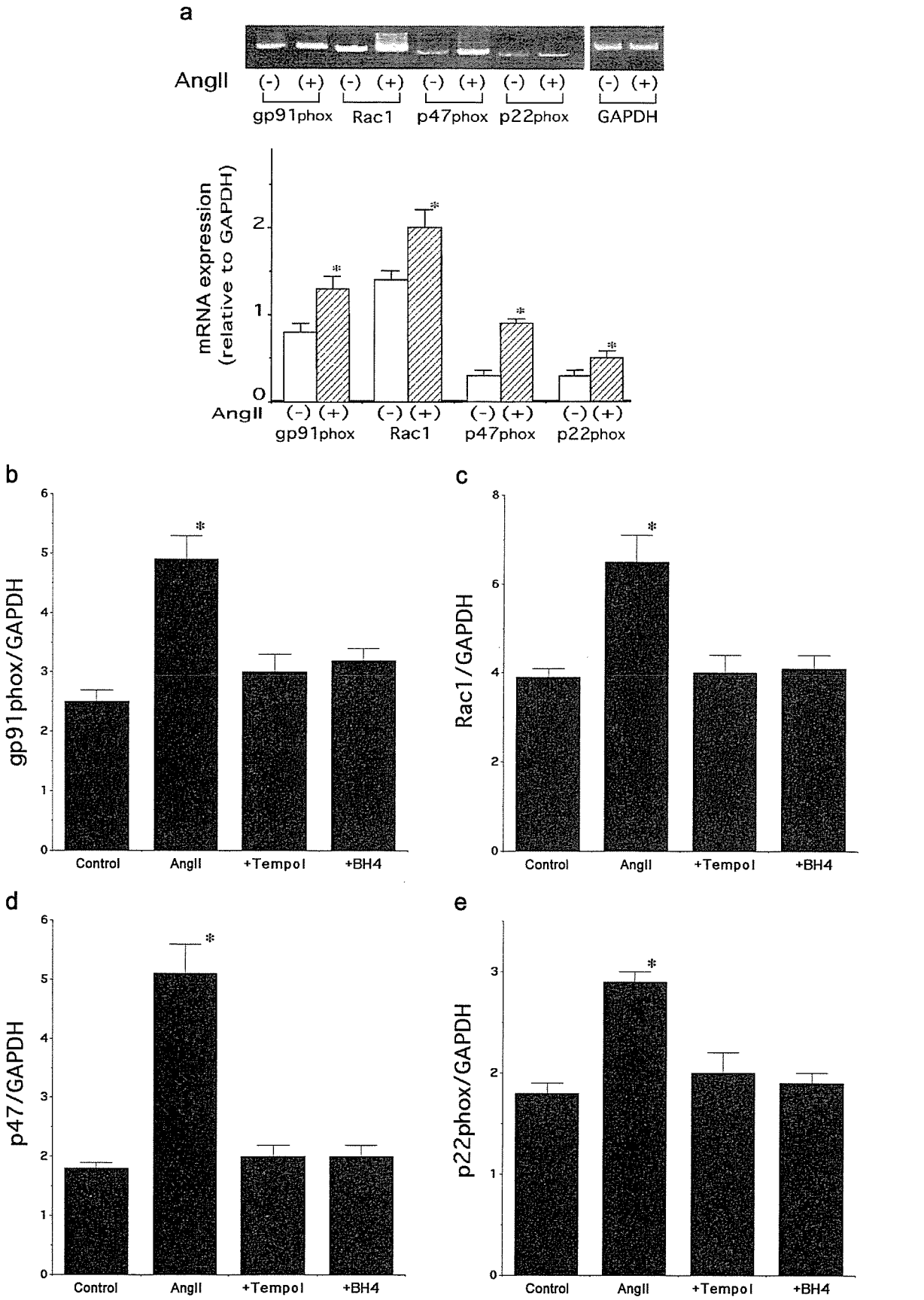

Fig. 4 **a** Expression of p22phox, gp91phox, p47phox and Rac1 in adipose tissue as assessed by RT-PCR in control rats and AngII-infused rats. **b–e** Expression of the NAD(P)H oxidase subunits gp91phox (**b**), Rac1 (**c**), p47phox (**d**), and p22phox (**e**) as evaluated by real-time PCR. Expression of the subunits was increased in AngII-infused rats, and treatment with tempol or BH4 significantly suppressed their upregulation. The results are expressed as means±SEM (n=4). *p<0.01 vs the control value

Angiotensin-II-induced superoxide production Low levels of superoxide were produced in vitro by aortic rings from control rats (Fig. 2). Infusion of AngII for 7 days produced a sixfold increase in vascular superoxide production, which was normalised by endothelial denudation (data not shown). Apocynin, an NAD(P)H oxidase inhibitor, markedly inhibited endothelial superoxide production in AngII-infused animals (Fig. 2). Similarly, cotreatment with tempol or BH4 significantly suppressed the AngII-induced production of superoxide anions. These results suggest that AngII induces endothelium-dependent superoxide production, predominately through NAD(P)H oxidase.

Plasma adiponectin levels and adiponectin mRNA levels in adipose tissue The AngII group had a significantly lower plasma adiponectin level than the control group (2.35±0.24 vs 5.60±0.44 μg/ml, $p<0.005$); however, concomitant treatment with tempol or BH4 restored plasma adiponectin concentrations (Fig. 3a).

Abundant adiponectin mRNA was detected by Northern blot analysis in abdominal adipose tissue from control (vehicle-infused) rats. Infusion of AngII reduced adiponectin mRNA levels by ≈50% (Fig. 3b), and concomitant treatment with tempol or BH4 prevented this reduction (Fig. 3c).

Angiotensin-II-induced upregulation of NAD(P)H oxidase The expression of mRNAs encoding p22phox, gp91phox, p47phox and Rac1 was significantly higher in the adipose tissue of rats in the AngII group than in the adipose tissue of the control rats (Fig. 4a). This increase was suppressed by concomitant treatment with tempol or BH4 (Fig. 4b–e). Treatment with tempol or BH4 alone had no effect on the expression of the transcripts for the NAD(P)H oxidase subunits.

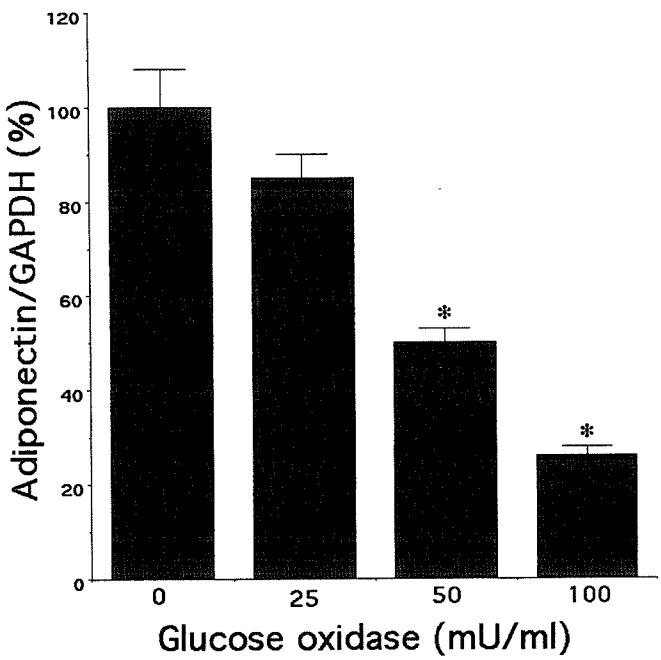

Fig. 5 Adiponectin mRNA levels in adipocytes exposed to H_2O_2. Fully differentiated 3T3-L1 adipocytes were serum-starved for 6 h and then exposed to H_2O_2 generated by adding different concentrations of glucose oxidase to the medium for 16 h. Total RNA was isolated from the cells and reverse transcribed. Quantification of adiponectin was performed by real-time PCR. The results are expressed as means±SEM (n=4). * $p<0.01$ vs the control value

Adiponectin mRNA expression in adipocytes following exposure to H_2O_2 To determine whether oxidants mediate the AngII-induced downregulation of adiponectin expression, we investigated this effect of AngII on adipocytes in culture following exposure to oxidative stress. Fully differentiated 3T3-L1 adipocytes were continuously exposed to H_2O_2 by the addition of glucose oxidase to the culture medium. Adiponectin mRNA levels were quantified by real-time PCR 16 h after exposure to H_2O_2. As shown in Fig. 5, glucose oxidase reduced adiponectin mRNA levels in a concentration-dependent manner. Given that cell respiration, measured by the MTT assay, was not significantly diminished even at the highest concentration of glucose oxidase used (data not shown), the reduction of mRNA by H_2O_2 cannot be explained by cytotoxicity.

Discussion

This study is the first to report hypoadiponectinaemia in a mammal as a consequence of chronic in vivo exposure to AngII. The results suggest a causal relationship between the AngII-mediated upregulation of NAD(P)H oxidase (with a resulting increase in ROS) and the impairment of adiponectin production. To the best of our knowledge, this is the first study to implicate a role for oxidative stress in the pathogenesis of hypoadiponectinaemia.

Clinical and laboratory studies have demonstrated that endothelial dysfunction is an important early step in atherosclerosis [36]. The endothelial dysfunction associated with long-term AngII treatment is primarily caused by an increase in NAD(P)H-oxidase-mediated vascular superoxide production [37–39]. The finding that tempol and BH4 restore endothelial function confirms that this is the case [25–28].

The key finding in the present investigation was that AngII infusion decreases circulating levels of adiponectin and reduces the expression of adiponectin mRNA in adipose tissue, the primary source of this adipokine. Suppression of adiponectin gene expression was prevented in AngII-infused rats by cotreatment with tempol or BH4, suggesting the involvement of ROS. Since adiponectin has multiple vasoprotective actions [6–10], decreased plasma adiponectin levels during AngII infusion may contribute to endothelial dysfunction, insulin resistance and cardiovascular pathophysiology. The suppression of adiponectin by AngII may be attributed to the upregulation of NADPH oxidase in adipose and vascular tissues, leading to the production of superoxide and derived species. We consider the increased production of superoxide anions to be primarily caused by the AngII-induced upregulation of NAD(P)H oxidase subunits, because it has been demonstrated that AngII-induced NAD(P)H oxidase activation is closely coupled to the increased expression of the enzyme in rats [40].

Support for the view that ROS can directly suppress adiponectin gene expression was provided by the results of our cell culture studies. Fully differentiated 3T3-L1 adipocytes were continuously exposed to H_2O_2, generated by glucose oxidase supplementation of the culture medium. At the highest concentration of glucose oxidase tested, it is estimated that cells may be exposed to concentrations of H_2O_2 of up to ~25 μmol/l [35]. This H_2O_2 exposure resulted in a concentration-dependent, substantial reduction in adiponectin mRNA expression. This finding reveals that oxidative stress within adipose tissue is sufficient to trigger hypoadiponectinaemia. The molecular mechanisms by which H_2O_2 and perhaps other ROS mediate the suppression of adiponectin mRNA levels await elucidation; diminished adiponectin gene transcription and accelerated adiponcectin mRNA degradation are viable possibilities.

Blockade of the AT1 receptor and inhibition of ACE both increase plasma levels of adiponectin. As demonstrated in the present study, AngII decreases circulating levels of adiponectin in vivo [41, 42], but does not regulate adiponectin levels in 3T3-L1 adipocytes in vitro [43]. Increased expression of NAD(P)H oxidase and increased ROS production might be localised to macrophages that invade adipose tissue, at least in obese animals, and this may be because of communication between macrophages and adipocytes in vivo.

Increasing evidence suggests that AngII is involved in the pathogenesis of a wide spectrum of cardiovascular diseases and insulin resistance [19, 37]. The present study demonstrates that oxidative stress induces hypoadiponectinaemia. Adiponectin levels are decreased in patients with obesity, diabetes and coronary artery disease. Obesity may result in increased oxidative stress in accumulated fat tissue, and patients with diabetes and coronary artery disease have high levels of oxidative stress. Thus, in addition to treating the underlying disease, it may be important to reduce oxidative stress to restore adiponectin levels and vascular integrity.

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