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Hypoadiponectinemia is caused by chronic blockade of nitric oxide synthesis in rats

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Abstract

Adiponectin is an adipocyte-derived anti-atherogenic protein. Adiponectin levels a[illegible] decreased i[illegible] patients and animal models with obesity, diabetes, and coronary artery disease. However, the mechanism by which adi[illegible]n levels are [illegible]d remains unknown. Since hypoadiponectinemia is closely linked to endothelial dysfunction, we examined th[illegible] regula[illegible] of adiponectin in a rat model of chronic blockade of nitric oxide (NO) synthesis by N^{ω}-nitro-L-arginine methyl ester (L-NAME). D[illegible]eased production of NO and increased production of O_2^- were observed in aorta from L-NAME-treated rats. Plasm[illegible]adiponectin levels a[illegible]adiponectin mRNA levels of adipose tissue were markedly decreased in L-NAME-treated rats. Cotreatment of [illegible]oglitazone (PIO) or allopurinol (ALL) with L-NAME restored plasma adiponectin concentration and fat adiponectin mRNA levels to co[illegible]ol levels. Thus, adiponectin levels were decreased in L-NAME-treated rats, however, they returned to normal following administration o[illegible]PIO due to tr[illegible]scriptional activation of the adiponectin gene, as well as administration of ALL, likely due to elimination of oxidativ[illegible]tress. Oxi[illegible]tive stress appears to be an important cause of hypoadiponectinemia.

1. Introduction

Adiponectin is abundant in human plasm[illegible] and decreased levels are observed in patients a[illegible] animal [illegible]odels with obesity, diabetes, and coronary [illegible] disease [1-[illegible]. Adiponectin has a number of vasc[illegible]ar pro[illegible]tive effects [6-10]. These findings suggest th[illegible] decreased p[illegible]sma adiponectin levels in the context of [illegible]besity and diabete[illegible]ay contribute to vascular disease i[illegible] these p[illegible]ents. However, the mechanism by which adip[illegible]ectin levels are decreased remains unknown. Vas[illegible]ar end[illegible]elial dys[illegible]nction plays a pivotal role in the [illegible]thoge[illegible]esis of [illegible]her[illegible]clerosis and enhances the risk of f[illegible]ure cardi[illegible]vascular e[illegible]nts [11,12]. Adiponectin has been sh[illegible]n to [illegible]itric oxide (NO) production in vascular en[illegible]helial cells [13,14]. A strong link between hypoadiponect[illegible]emia and endothelial dysfunction has been reported in man [15,16]. Thus, the established relationship between insulin resistance and vascular endothelial cell dysfunction might be partly explained by decreased levels of adiponectin. Therefore, it is important to investigate the regulation of adiponectin levels in man or animals with endothelial dysfunction. The present study examined plasma adiponectin levels in a rat model with blockade of NO synthesis by administration of an inhibitor of NO synthesis, N^{ω}-nitro-L-arginine methyl ester (L-NAME). We found decreased plasma levels of adiponectin in rats treated with L-NAME. To elucidate the mechanism of the observed effects of L-NAME, we then examined the expression of adipose tissue adiponectin mRNA in these rats. We also examined whether antioxidant drugs might reverse the effects of L-NAME on decreased adiponectin levels.

2. Materials and methods

2.1. Animal model of inhibition of NO synthesis

The present experiments were reviewed and approved by the Committee on Ethics of Animal Experiments, and were conducted according to the Guidelines for Animal Experiments, at Dokkyo University Faculty of Medicine.

Fourteen-week-old male Wistar-Kyoto rats were housed singly in a pyrogen-free facility. Four groups of rats were studied. The first (control) group received untreated laboratory chow and drinking water. The second group (L)

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received L-NAME in the drinking water (1 mg/mL). At this concentration, the daily intake of L-NAME was 100 mg/kg per day [17,18]. The third group (P) received pioglitazone (20 mg/kg) in the chow. The fourth group (L+A) received L NAME in the drinking water and allopurinol by intraperitoneal injection (30 mg/kg per day).

2.2. Vessel Harvesting and preparation

On day 5 of treatment, we measured heart rate as well as systolic blood pressure by the tail-cuff method. The rats were anesthetized 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 removed rapidly, and the vessel lumen was flushed with the solution. In some rats, the aorta was cut into three 5-mm ring segments for use in studies of NO production, as well as superoxide anion production.

2.3. Measurement of NO production

The 5-mm ring segments of the aorta were incubated in 2 mL of Hanks' balanced salt solution containing a calcium ionophore A23187 (1 μmol/L) and L-arginine (100 μmol/L), as previously described. A chemiluminescence-based NO analyzer (270B, Sievers) was used to measure NO production. Specific NO-generating capacity was expressed as nanomoles per hour per dry weight.

2.4. Measurement of plasma NO

Plasma nitrite and nitrate levels (NO_2^- and NO_3^-) were measured with an automated NO detector/high-performance liquid chromatography system (ENO10, Eicom Co, Kyoto, Japan). Nitrite and nitrate in the dialysate were separated using a reverse-phase separation column, and nitrate was reduced to nitrite in a reduction column. Nitrite was mixed with a Griess reagent, after which the absorbance at 540 nm was measured by flow-through spectrophotometry.

2.5. Measurement of vascular superoxide anion production

Superoxide anion production was measured using lucigenin chemiluminescence, as previously described [19]. Briefly, the thoracic aortas were carefully dissected and cleared of perivascular tissue and blood contaminants under a microscope, after which they were placed in HEPES-buffered physiological salt solution (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, and glucose 5.8, aerated with 95% O_2 and 5% CO_2). In a preliminary study, we confirmed no adhesion of inflammatory cells to the endothelium (data not shown). Scintillation vials containing 1 mL HEPES-buffered PSS with 5 μmol/L lucigenin (bis-N-methylacridinium nitrate) were placed into a scintillation counter (Luminescence Reader BLR 301, ALOKA, Tokyo, Japan). We used Tiron (10 mmol/L; Sigam, Tokyo, Japan), a superoxide scavenger, in all experiments to confirm the validity of our technique with lucigenin. After dark adaptation, background counts were recorded for 3 minutes and then 3 vascular segments (5 mm in length) from each thoracic aorta were added to the vial. Scintillation counts were then recorded every minute for 10 minutes and the respective background counts were subtracted. The vessels were then dried for determination of dry weight. Lucigenin counts were expressed as counts per minute per milligram of dry weight.

2.6. Measurement of adiponectin levels in plasma

The plasma concentration of adiponectin was determined by ELISA using a kit for measurement of rat/mouse adiponectin (Otsuka Pharmaceuticals, Tokyo, Japan).

2.7. Measurement of adiponectin mRNA levels in adipose tissue

Standard Northern blotting was used to investigate the mRNA expression of adiponectin in adipose tissue, as previously described [20]. After probing for adiponectin, filters were stripped and re-probed for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Expression of eNOS, iNOS, p22phox, gp91phox, p47phox, Rac1, and GAPDH mRNA was also analyzed by reverse transcription-polymerase chain reaction (RT-PCR), as previously described [20].

2.8. Statistical analysis

Data are expressed as mean values ± SEM. Differences between two experiments were compared by Student *t* tests. Differences between three experiments were determined by 2-way ANOVA and Bonferroni's multiple comparison test. A *P* value of .05 was considered statistically significant.

3. Results

3.1. Body weight and hemodynamic parameters

During the 5-day treatment period, body weights in the control and L groups did not change, and weights did not differ significantly between the 2 groups. During

Table 1

Groups	Body weight, g	Systolic blood pressure, mmHg	Heart rate, bpm
Control group (n = 9)			
Day 0	302 ± 10	126 ± 11	398 ± 11
Day 5	313 ± 12	130 ± 8	402 ± 12
L group (n = 9)			
Day 0	312 ± 7	128 ± 9	408 ± 15
Day 5	314 ± 12	169 ± 13*,**	339 ± 11*,**

Data are mean ± SE.
* $P < 0.01$ vs control group.
** $P < 0.01$ vs day 0.

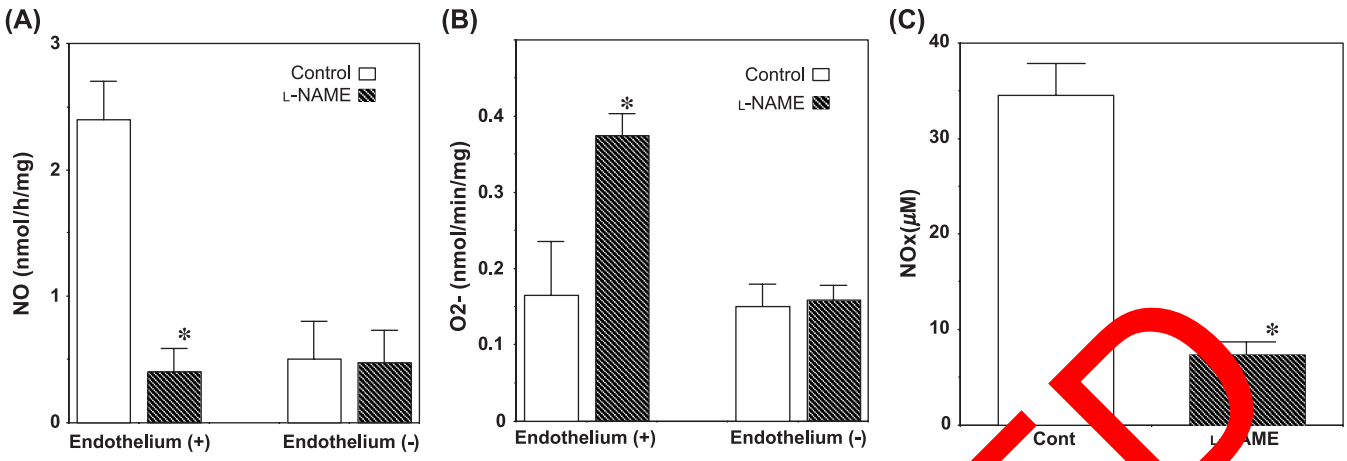

Fig. 1. NO (A) (nanomoles per hour per milligram tissue) and O_2^- (B) (nanomoles per 10 minutes per milligram tissue) production in [illegible] segments and plasma NOx concentration (C) in rats. *$P < .01$ vs control group. Each bar represents n = 6. NOx indicates nitrate + nitrite.

this period, the L group exhibited a significant rise in systolic arterial pressure, compared with the control group (Table 1). While heart rate did not change significantly in the control group, a reduction in heart rate was seen in the L group.

3.2. Aortic NO and O_2^- production and plasma NOx concentration

NO production was much lower in the L group than in the control group (Fig. 1A). Removal of the endothelium markedly decreased aortic NO production in the control group to the same level as the L group with intact endothelium (Fig. 1A). Removal of the endothelium did not affect NO production in the L group.

Production of O_2^- in the aortic segments with intact endothelium was greater in the L group than in the control group (Fig. 1B). In the L group segments without endothelium, O_2^- production was similar to that of the control group segments without endothelium (Fig. 1B).

Plasma NOx was markedly reduced in the L group, compared with the control group (Fig. 1C).

3.3. Plasma adiponectin and adiponectin mRNA levels in adipose tissue

Plasma adiponectin in the control group was 5.90 ± 0.64 μg/mL, while that of the L group was significantly lower at [illegible] ± 0.42 μg/mL ($P < .005$) (Fig. 2A).

There was an abundance of adiponectin mRNA in the abdominal adipose tissue of control rats, while mRNA expression was clearly reduced in the L group (Fig. 2B).

There observed no difference of mRNA expression in eNOS, p22phox, gp91phox, p47phox, and Rac1 in adipose tissue between the control and the L group. iNOS mRNA did not expressed in adipose tissue from the control and the L group.

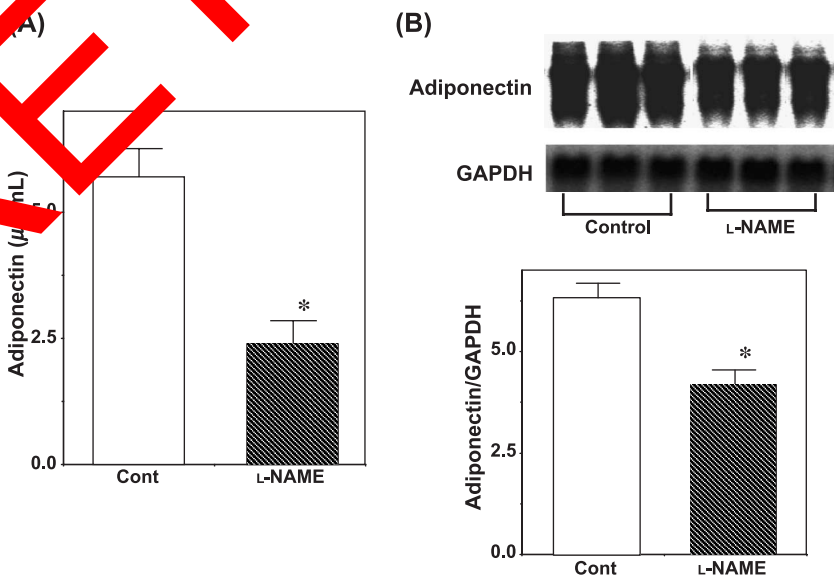

Fig. 2. Plasma adiponectin concentration (n = 8) (A) and adiponectin mRNA levels in adipose tissue (n = 3) (B). *$P < .01$ vs control group.

3.4. Effect of pioglitazone and allopurinol on plasma adiponectin concentration and adiponectin mRNA levels in adipose tissue

Plasma adiponectin levels were significantly lower in the L group. To examine the effects of pioglitazone and allopurinol on plasma adiponectin levels, pioglitazone or allopurinol was administered to rats with and without L-NAME for 5 days. Pioglitazone increased rat adiponectin levels, while no changes were observed with allopurinol. However, both pioglitazone (L+P) and allopurinol (L+A) restored rat adiponectin levels to control levels in rats treated with L-NAME (Fig. 3A). Similarly, pioglitazone and allopurinol recoverd adiponectin mRNA levels in adipose tissue from L-NAME-treated rats (Fig. 3B and C).

4. Discussion

This study showed reduced plasma adiponectin levels in an animal model of inhibition of NO synthesis. Adiponectin mRNA levels were also reduced, suggesting that the observed reduction in adiponectin l[illegible]as due to decreased production. The recovery [illegible] adipon[illegible]in levels in

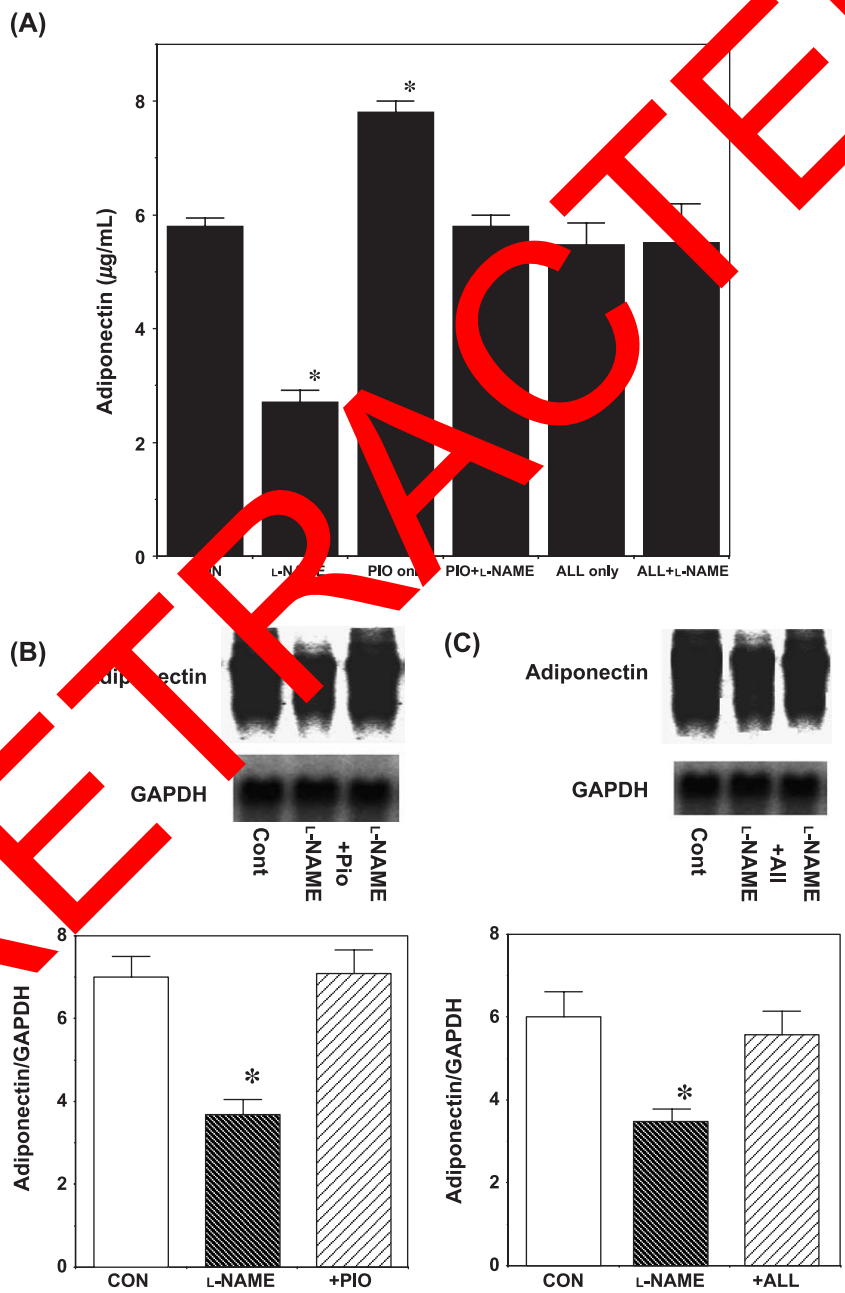

Fig. 3. Effects of pioglitazone and allopurinol on plasma adiponectin concentration (n = 8) (A) and adiponectin mRNA levels in adipose tissue (B and C). *$P < .01$ vs control group.

animals treated with pioglitazone and allopurinol suggests that stimulation of adiponectin production and/or a reversal of inhibition of adiponectin production might occur, thereby reversing the decline in adiponectin levels. Importantly, this suggests that oxidative stress might reduce adipopectin production.

It has been show that peroxisome proliferator-activated receptor (PPAR)-γ plays a significant role in transcriptional activation of the adiponectin gene via PPAR response element (PPRE) and its promoter [21]. Indeed, treatment with PPAR-γ agonists clearly increases adiponectin levels in humans [22,23]. We confirmed that pioglitazone increases adiponectin levels and allows recovery of adiponectin levels to control levels in L-NAME-treated rats. This might be due to transcriptional activation of the adiponectin gene in adipose tissue. This normalization of adiponectin levels by pioglitazone in L-NAME-treated rats might explain the results of previous reports in which prevention of L-NAME-induced coronary inflammation and arteriosclerosis by pioglitazone was observed [24].

Oxidative stress appears to reduce adiponectin levels. We confirmed decreased adiponectin levels in L-NAME-treated rats, as well as in rats infused with angiotensin II (unpublished data). In both groups of rats, hypertensive and cardiovascular effects were observed due to oxidative stress. Insulin resistance was also induced in both groups of rats [25,26]. Treatment of the animals with antioxidant drugs resulted in partial to total recovery of impairment [27,28]. Thus, reduced adiponectin production might play a pathogenic role in the development of cardiovascular effects in these rats. Furthermore, reduced adiponectin levels are closely associated with oxidative stress.

We examined the mRNA levels of NADPH oxidase components, as well as NO synthases, in the adipose tissue of L-NAME-treated rats in order to clarify the role of oxidative stress in fat, which is the source of adiponectin. Angiotensin II activation and angiotensin converting enzyme activation have been demonstrated in L-NAME treated rats [27,29], thus suggesting a pathogenic role of oxidative stress. Angiotensin II is thought to induce oxidative stress mainly via induction/activation of NADPH oxidase [30,31]. However, we did not observe an increase in the mRNA levels of NADPH oxidase components or NO synthases in adipose tissue from L-NAME treated rats, even though increased mRNA levels were observed in angiotensin II-treated rats (data not shown). Furthermore, allopurinol recovered mRNA levels in L-NAME treated rats, indicating that xanthine oxidase, rather than NADPH oxidase or uncoupled NOS, might play a role in the L-NAME-mediated induction of oxidative stress in rat adipose tissue.

Our present findings suggest that oxidative stress reduces adiponectin production. Adiponectin has been shown to suppress proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL [32]. It has also been demonstrated that angiotensin II-induced apoptosis in human endothelial cells is inhibited by adiponectin through restoration of the association between endothelial nitric oxide synthase and heat shock protein 90 [33]. Adiponectin levels are decreased in patients and animal models with obesity, diabetes, and coronary artery disease [1-5], in which oxidative stress appears to affect adiponectin production in adipose tissue. Thus, relief of oxidative stress, requiring different strategies depending on the underlying cause, might have a role in the treatment of these diseases by normalizing adiponectin levels and thus preventing associated pathology.

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