Endothelium-specific activation of NAD(P)H oxidase in aortas of exogenously hyperinsulinemic rats

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Kashiwagi, Atsunori, Kazuya Shinozaki, Yoshihiko Nishio, Hiroshi Maegawa, Yasuhiro Maeno, Akio Kanazawa, Hitoheto Kojima, Masakazu Haneda, Hideki Hidaka, Hitoshi Yasuda, and Ryuichi Kikkawa. Endothelium-specific activation of NAD(P)H oxidase in aortas of exogenously hyperinsulinemic rats. Am. J. Physiol. Environ. 40: E976–E983, 1999.—To examine the effects of chronic hyperinsulinemia on vascular tissues, we examined the production of superoxide anion (O2•−) in the aortic tissues of control and exogenously hyperinsulinemic rats performed by the implantation of an insulin pellet for 4 wk. O2•− production by aortic segments from hyperinsulinemic rats was 2.4-fold (lucigenin chemiluminescence method) and 1.7-fold (cytochrome c method) of that of control rats without any differences in O2•− degrading activities in aortic tissues, respectively (P < 0.025). The increment was completely abolished in the presence of either 100 μmol/l apocynin (an inhibitor of NADPH oxidase) or 10 μmol/l diphenyleneiodo- niun (an inhibitor of flavin-containing enzyme) and was exclusively endothelium dependent. Consistently, NAD(P)H oxidase activities in endothelial homogenate in hyperinsulinemic rats were dose dependently stimulated above the values of control rats, although these activities in nonendothelial homogenate were not significantly stimulated by insulin. Furthermore, an insulin effect was also demonstrated 1 h after exposing aortic tissues to insulin. These results indicate that O2•− production specifically increases in endothelium of aortic tissues in chronic hyperinsulinemic rats through the activation of NAD(P)H oxidase.

oxidative stress; free radicals; endothelial cells

PREVIOUS EPIDEMIOLOGICAL studies indicate a close relationship between plasma insulin levels and the incidence of cardiovascular disease, supporting the idea that hyperinsulinemia is involved as an atherogenic factor (25, 32). Stout (30) and other researchers (20) have raised the possibility that chronic hyperinsulinemia may contribute to the development of atherosclerosis via the direct effect of insulin on arteries. The following studies also show that insulin increases both the synthesis and release of vasoactive substances related to atherogenesis (6, 19, 23).

In contrast, based on the recent epidemiological studies, the role of hyperinsulinemia itself as an atherogenic factor has been questioned (9) because hyperinsulinemia as a result of insulin resistance in patients is strongly linked to the coexistence of multiple cardiovascular risk factors, including essential hypertension, obesity, impaired glucose tolerance, and dyslipidemia (27). In terms of the biological effects of insulin on vascular cells, insulin has been shown to have an acute vasodilatory effect through endothelial nitric oxide (NO) production, and therefore insulin produces an antiatherogenic effect (1). On the other hand, insulin stimulates NADPH-dependent H2O2 generation in human adipocyte plasma membrane (10). Although there is no study concerning insulin effect on superoxide anion (O2•−)-producing in vascular tissues, it also has been reported that vasoactive peptides can release O2•− through various O2•−-producing systems such as NAD(P)H oxidase (7, 26, 31) and lipoxygenase (21), resulting in an atherogenic insulin on vascular tissues. Thus increased O2•− production is one of possible mechanisms involved in impairing vascular dilatation through scavenging NO (22), as well as the impaired synthesis release of NO (24, 29). Therefore, it is worthwhile to evaluate the significance of hyperinsulinemia itself on O2•− production in vascular tissues in experimental animals. To study the direct effect of high-insulin concentrations on vascular cell function, we created a hyperinsulinemic condition in rats that were not insulin resistant by the subcutaneous implantation of an insulin pellet.

Thus the purpose of this study was to determine whether O2•− production increased in aortic segments of exogenously hyperinsulinemic rats without insulin resistance. Furthermore, we tried to characterize the specific vascular cell type that released O2•− and further attempted to identify the activated oxidase species in aortic homogenates in the presence or absence of endothelium of both control and hyperinsulinemic rats that were induced by the subcutaneous implantation of an insulin pellet.

MATERIALS AND METHODS

Materials. Insulin pellets were obtained from Linshin Canada. Concanaavalin A-Sepharose was obtained from Amerham Pharmacia Biotech. All other materials were purchased from Sigma Chemical (St. Louis, MO).

Animals. Six-week-old male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 150 g were housed in an environmentally controlled room with 12:12-h light-dark cycle and free access to laboratory chow and water. The animals were divided into two groups; 1) control and 2) hyperinsulinemic rats induced by implantation of an insulin pellet (insulin treated). Those animals were fed on a diet (Oriental Yeast, Tokyo, Japan) consisting of 58% carbohydrate (no fructose), 12% fat, and 30% protein (calorie %). For the continuous delivery of insulin, an incision was made in
the midscapular region under pentobarbital anesthesia (50 mg/kg body wt) and either one-half or one piece of insulin pellet was implanted (release rate of 0.5 U/day and 1.0 U/day, respectively) into the back of the rats for 2 and 4 wk, respectively. Blood pressure was measured the day before the experiment, and the rats had been trained with the apparatus three times before measurement. Both systolic blood pressure and diastolic blood pressure in the tail region were measured using an electrophysymomanometer after the rat was prewarmed for 15 min (2).

Assessment of in vivo insulin action. Insulin sensitivity was measured by the steady-state plasma glucose method using somatostatin, originally described by Harano et al. (8). After an overnight fast, rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg body wt) and the right jugular vein was exposed and cannulated with a polyethylene tube for the administration of the infusate. Rats were administered with the infusate containing somatostatin (120 mg·kg⁻¹·h⁻¹), glucose (1.0 g·kg⁻¹·h⁻¹), and insulin (2.0 U·kg⁻¹·h⁻¹, human actrapid, NovoNordisk, Denmark) at a flow rate of 2.8 ml/h for 120 min. Blood samples for determining plasma glucose levels were obtained from the tail vein at 0, 30, 60, 90, and 120 min after the initiation of the infusion. The steady-state plasma glucose and insulin levels were measured at 120 min after the initiation of infusion.

Preparation of aortic tissues. After 2- or 4-wk insulin infusion, control and insulin-treated rats were killed under pentobarbital anesthesia. The thoracic aortas (0.6–0.8 cm outside diameter) were isolated and carefully cut into strips to preserve the endothelium. In some strips, the endothelium was removed by gentle rubbing of the intimal surface with a cotton ball. Blood samples for measurements of plasma glucose, lipid, and insulin concentrations were obtained from the tail vein. Blood glucose concentration was determined by glucose oxidase method, and serum total cholesterol, triglyceride, and free fatty acid concentrations were measured by standard enzymatic methods. Plasma insulin concentration was measured by radioimmunoassay using the rat insulin RIA kit (DiaSorin, Stillwater, MN).

Measurements of ex vivo aortic O₂ production. O₂ production in aortic segments was measured using lucigenin-enhanced chemiluminescence described as previously (22). This methodology has been proven to be quite specific for O₂ detection (18) and useful in studies of vascular O₂ production. Segments of thoracic aortas (20 mm) were isolated, placed in a modified Krebs-HEPES buffer (pH 7.4) containing (in mmol/l) 99.0 NaCl, 4.69 KCl, 1.87 CaCl₂, 1.20 MgSO₄, 1.03 K₂HPO₄, 25 NaHCO₃, 20 Na-HEPES, and 11.1 glucose, and then allowed to equilibrate for 30 min at 37°C. After 5 min of dark adaptation, scintillation vials containing 2 ml Krebs-HEPES buffer with 50 µmol/l lucigenin were placed into a scintillation counter (Tri-Carb 1500; Packard Instrument, Meriden, CT) switched to the out-of-coincidence mode. Chemiluminescence values were obtained at 30-s intervals over 15 min, and readings in each of the last 10 min were averaged. The vessel was dried at 90°C for 24 h, and then the dry weight was measured. Lucigenin count was expressed as counts per minute per milligram of the dry weight of vessel. Background counts were determined by vessel-free incubations and subtracted from vessel readings.

Some vessels were incubated for 30 min with either 100 µmol/l apocynin (an inhibitor of activity assembly of the components of NADPH oxidase), 10 µmol/l diphenyleneiodonium (an inhibitor of flavin-containing enzymes), 100 µmol/l oxypheni- nol (an inhibitor of xanthine oxidase), 100 µmol/l rotenone (an inhibitor of mitochondrial respiration), 10 µmol/l indometha- cin (an inhibitor of cyclooxygenase), 10 µmol/l nordihydro-
agents exaggerates any electron leak contribution from mitochondrial sources. $O_2$ production in response to NADH or NADPH was also examined after the addition of apocynin (100 µmol/l), diphenyleneiodonium (10 µmol/l), indomethacin (10 µmol/l), NDGA (10 µmol/l), or L-NNA (1 µmol/l). Values were standardized to the amount of protein present. Similarly, the $O_2$ production by those enzyme reactions was also determined by SOD-inhibitable reduction of succinylated cytochrome c (12). Protein content was measured by the Lowry method (16).

Vascular $O_2$ scavenging activity. Vascular $O_2$-scavenging activity was spectrophotometrically measured by modification of the method originally described by Salin and McCord (28). Segments of the thoracic aortas were harvested as previously described and homogenized in PBS (2 ml) with a homogenizer, the homogenates were centrifuged at 13,600 g for 15 min, and the supernatant was kept on ice until analysis. The aliquot (0.1 ml) was incubated with 100 µmol/l xanthine, 15 µmol/l cytochrome c (horse heart type IV), 20 mmol/l NaHCO$_3$, 1.0 mmol/l NaN$_3$, and 0.1 mmol/l EDTA. The assay was initiated by the addition of 0.025 ml xanthine oxidase (1.0 U/ml), and the resultant superoxide generation was measured by the increment of absorbance at 550 nm over the 20-s reaction time. The enzyme activity was calculated from a linear dose-response curve obtained using 0.1–10 U/ml bovine erythrocyte Cu,Zn-SOD and expressed as units of SOD normalized based on its protein content. The residual Cu,Zn-SOD-independent superoxide-scavenging activity was determined from identically prepared vessels that were incubated with 10 mmol/l diethyldithiocarbamate (an inhibitor of Cu,Zn-SOD) for 30 min.

The extracellular SOD (EC-SOD) activity was spectrophotometrically measured by a previously reported method (17). The tissues were homogenized in 10 vol of 50 mmol/l sodium acetate (pH 5.5) containing 0.3 mol/l KBr, and the aliquots were centrifuged at 20,000 g for 15 min. The resultant supernatants (0.61–0.83 ml) were applied on a concanavalin A-sepharose column (column vol 0.5 ml), which was equilibrated with 10 mmol/l potassium-phosphate buffer containing 120 mmol/l NaCl. Sample aliquots (0.5 ml) were applied to allow binding to the lectin. After 5 min, a 2-ml phosphate buffer was added. The eluate contained both Cu,Zn-SOD and Mn-SOD. Then the column was washed with 10 ml of the phosphate buffer. The EC-SOD was then eluted with 2 ml of 0.5 mol/l α-methyl α-mannoside in 50 mmol/l sodium phosphate (pH 6.5). SOD was determined based on its ability to catalyze the disproportionation of $O_2$ in an alkaline aqueous solution. The disproportionation was spectrophotometrically measured as previously described (17). One unit in the assay is defined as the activity that brings about a decay in $O_2$ concentration at a rate of 0.1 s$^{-1}$ in 3 ml buffer.

Statistical analysis. All values were presented as means ± SE. Differences between the two groups were evaluated by Student’s t-test, and comparison among three groups were performed using ANOVA with a post hoc Scheffé’s comparison. $P < 0.05$ was considered significant.

RESULTS

Metabolic characteristics and blood pressure of the rats. As shown in Table 1, control and insulin-treated (0.5 and 1.0 U/day) rats gained weight to a similar degree over the study period. Animals receiving insulin infusion (0.5 and 1.0 U/day) for 4 wk demonstrated significant decreases in plasma glucose levels and significant increases in plasma insulin levels compared with control rats. However, there were no differences in

### Table 1. Characteristics of experimental animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.5 U/day</th>
<th>1.0 U/day</th>
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</thead>
<tbody>
<tr>
<td>Baseline data, n = 5/group</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Weight, g</td>
<td>320 ± 6</td>
<td>337 ± 13</td>
<td>332 ± 11</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>5.48 ± 0.18</td>
<td>4.87 ± 0.24$^a$</td>
<td>4.40 ± 0.26$^c$</td>
</tr>
<tr>
<td>Insulin, pmol/l</td>
<td>119 ± 8</td>
<td>256 ± 24$^b$</td>
<td>568 ± 34$^d$</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>1.70 ± 0.09</td>
<td>1.62 ± 0.11</td>
<td>1.74 ± 0.12</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>1.33 ± 0.19</td>
<td>0.90 ± 0.25</td>
<td>0.50 ± 0.13</td>
</tr>
<tr>
<td>Free fatty acid, mmol/l</td>
<td>0.15 ± 0.02</td>
<td>0.20 ± 0.04</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117 ± 2.4</td>
<td>120 ± 3.7</td>
<td>114 ± 4.1</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>71 ± 3.5</td>
<td>60.2 ± 4.7</td>
<td>73 ± 6.2</td>
</tr>
</tbody>
</table>

The values are means ± SE, n = no. rats. $^a$$P < 0.05$, $^b$$P < 0.01$, $^c$$P < 0.001$, vs. control rats. $^d$$P < 0.05$ vs. insulin-treated (0.5 U/day) rats. SSPG, steady-state plasma glucose level; SSPI, steady-state plasma insulin level; ND, not determined.

blood pressure and serum total cholesterol, triglyceride, and free fatty acid levels between control and insulin-treated rats. Furthermore, chronic exogenous hyperinsulinemia induced by subcutaneous infusion of insulin did not impair the insulin effect on in vivo glucose utilization because the steady-state plasma glucose concentration in insulin-treated (1.0 U/day for 4 wk) rats measured by the glucose, somatostatin, and insulin infusion method was similar to that of the control rats (Table 1).

$O_2$ generation from aortas with or without endothelium. We measured the basal $O_2$ generation from aortas with or without endothelium in ex vivo conditions using the lucigenin chemiluminescence method. As shown in Table 2, $O_2$ production by aortic segments from insulin-treated (1.0 U/day) rats was 2.4-fold that in control rats ($P < 0.0001$). Endothelial removal produced about a 40% reduction ($P < 0.01$) of $O_2$ production. Thus, after removal of the endothelium, a reduction in $O_2$ production rate between the two groups was no longer apparent. Furthermore, incubation of intact aortic segments from the two groups with either 100 µmol/l apocynin (an inhibitor of NADPH oxidase) or 10 µmol/l diphenyleneiodonium [an inhibitor of NAD(P)H oxidase] for 30 min abolished the increment of $O_2$ production by insulin treatment. In contrast, neither oxyxynonol (a xanthine oxidase inhibitor), rotenone (an inhibitor of mitochondrial respiration), indomethacin (an inhibitor of cyclooxygenase), NDGA (an inhibitor of lipoxynase), nor L-NNA (an inhibitor of NO synthase) affected the lucigenin signal in intact aortic segments (Table 2).

These results indicate that basal $O_2$ production in aortic endothelium increased in insulin-treated rats at least through the activation of NAD(P)H oxidase.
Table 2. Superoxide anion production from aortic vessels incubated in absence or presence of various inhibitors

<table>
<thead>
<tr>
<th>Endothelium</th>
<th>Incubation Condition</th>
<th>n</th>
<th>Vascular Superoxide Production, counts/min × 10^4/mg of dry wt of vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>Buffer alone</td>
<td>4</td>
<td>10.5 ± 1.4 11.4 ± 0.4</td>
</tr>
<tr>
<td>+</td>
<td>Buffer alone</td>
<td>13</td>
<td>17.3 ± 1.1e 41.5 ± 4.3bd</td>
</tr>
<tr>
<td>+</td>
<td>Apocynin, 100 µmol/l</td>
<td>5</td>
<td>9.1 ± 0.3e 9.9 ± 0.7f</td>
</tr>
<tr>
<td>+</td>
<td>Diphenyleneiodonium, 10 µmol/l</td>
<td>5</td>
<td>6.9 ± 1.3e 8.6 ± 2.5f</td>
</tr>
<tr>
<td>+</td>
<td>Oxypurinol, 100 µmol/l</td>
<td>5</td>
<td>18.5 ± 1.1 39.8 ± 2.2f</td>
</tr>
<tr>
<td>+</td>
<td>Rotenone, 100 µmol/l</td>
<td>5</td>
<td>16.0 ± 0.3 36.4 ± 2.9f</td>
</tr>
<tr>
<td>+</td>
<td>Indomethacin, 10 µmol/l</td>
<td>5</td>
<td>16.8 ± 1.7 40.8 ± 4.6f</td>
</tr>
<tr>
<td>+</td>
<td>Nordihydroguaiaretic acid, 10 µmol/l</td>
<td>5</td>
<td>15.4 ± 2.1 38.1 ± 4.0f</td>
</tr>
<tr>
<td>+</td>
<td>N^G^N^O^2-nitro-arginine, 1 µmol/l</td>
<td>5</td>
<td>16.2 ± 0.4 35.7 ± 2.5f</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = no. rats. aP < 0.01, bP < 0.0001 vs. each vessel without endothelium incubated in buffer alone. cP < 0.01, dP < 0.0001 vs. corresponding control vessels with endothelium obtained from control rats. eP < 0.001, fP < 0.0001 vs. corresponding control vessels with endothelium incubated in buffer alone, using Student's unpaired t-test.

To confirm increased O_2 generation from aortic vessels in insulin-treated rats, we employed a different method for measuring O_2 production such as the cytochrome c method. As shown in Fig. 1, O_2 generation (nanomoles per minute per milligram dry weight of vessels) in the vessels of insulin-treated (1.0 U/day) rats was 2.48 ± 0.16, which was 68% (P < 0.025) higher than that of control vessels (1.48 ± 0.28).

Enzyme source for production of O_2 in vascular homogenates, O_2 production in response to the addition of a variety of substrates was examined in vascular homogenates. As shown in Fig. 2A, O_2 production derived from either NADPH or NADH oxidases was greater than that of the other potential substrates: arachidonic acid, succinate, or xanthine, respectively. Furthermore, the difference in the O_2 production in the presence or absence of the endothelium was not significant in control rats, although activities of both NADPH and NADH oxidases in the presence of endothelium tended to be greater than in the absence of endothelium. These results indicate that both activities in vessel homogenates of control rats were mainly derived from nonendothelial cells. However, in insulin-treated (1.0 U/day for 4 wk) rat vessels, O_2 production was from not only NADH oxidase, but also NADPH oxidase, in the presence of endothelium was significantly greater than that in the absence of endothelium, respectively (Fig. 2C). Similarly, chronic insulin treatment for 2 wk also significantly (P < 0.025) stimulated NADH oxidase activity (+ insulin 10,669 ± 1,837 vs. control 4,035 ± 840 counts·min⁻¹·mg protein⁻¹, n = 4 for each group) and NADPH oxidase activity (+ insulin 5,642 ± 692 vs. control 3,615 ± 723 counts·min⁻¹·mg protein⁻¹, n = 4 for each group) by 2.6-fold and 1.6-fold compared with control rat aortas, respectively. Furthermore, O_2 generation derived from NADPH and NADH oxidases in the absence of endothelium was not significantly different between control and 4-wk insulin-treated rats, respectively (Fig. 2, A and C). These results indicate that chronic insulin infusion for 4 wk results in enhanced NADPH and NADH oxidase activities only in the presence of endothelium. As shown in Fig. 3, consistent with increased O_2 generation in chronic hyperinsulinemic rat aortas in the ex vivo condition, NAD(P)H oxidase activities in aortic homogenates were also increased, depending on exogenous insulin doses, and those activities in endothelium of insulin-treated (1.0 U/day) rats were about fourfold greater than that of control rats, respectively. Consistently, the addition of NAD(P)H oxidase inhibitors (either 100 µmol/l apocynin or 100 µmol/l diphenyleneiodonium) significantly reduced lucigenin chemiluminescence in the presence of either 500 µmol/l NADH or 500 µmol/l NADPH in both control and insulin-treated (1.0 U/day) rats (data not shown). In contrast, O_2 generation from the vessel homogenates was not significantly affected in the presence of either arachidonic acid, succinic acid, or xanthine (Fig. 2). These results indicate that an increment in O_2 production was induced by insulin only through the activation of either NADH or NADPH oxidases.

To further confirm these increased activities of NADPH and NADH oxidases, we also measured these enzyme activities by the cytochrome c method. Consistent with results from using the lucigenin chemiluminescence method, NADPH oxidase activity in vessel homogenate of insulin-treated (1.0 U/day) rats (2.20 ± 0.17 nmol·min⁻¹·mg protein⁻¹) was 79% (P < 0.01) higher than that of control vessels (1.23 ± 0.19 nmol·min⁻¹·mg protein⁻¹; Fig. 4). Similarly, NADH
oxidase activity in vessel homogenate of insulin-treated (1.0 U/day) rats (2.76 ± 0.34 nmol·min⁻¹·mg protein⁻¹) was 116% (P<0.025) higher than that of control vessels (1.28 ± 0.26 nmol·min⁻¹·mg protein⁻¹; Fig. 4).

To measure the acute insulin effect on NAD(P)H oxidase activities in vascular homogenate, we incubated aortic segments in the presence of 10 nmol/l insulin for 1 h in ex vivo conditions and then measured these enzyme activities. Insulin stimulated NADH oxidase activity by 2.1-fold (P<0.01) compared with that of control rat aortas (from 3,146 ± 517 to 6,463 ± 717 counts·min⁻¹·mg protein⁻¹ for each group). Similarly, insulin also stimulated NADPH oxidase activity increased by 1.7-fold (P<0.03) compared with that of aortas in control rats (from 4,166 ± 606 to 7,044 ± 763 counts·min⁻¹·mg protein⁻¹ for each group).

Vascular scavenging activity of O₂⁺. The net vascular production of O₂⁺ is modified by local O₂⁻-scavenging activity. Arterial tissue contains considerable amounts of SOD that effectively converts superoxide to H₂O₂. In the present study, the activity of Cu,Zn-SOD, a major source of superoxide catabolism in arterial tissue, was not different between the two groups [control 7.69 ± 0.43 and insulin treated (1.0 U/day) 8.00 ± 0.18 IU/mg protein, n = 4 for each group]. After inhibiting Cu,Zn-SOD activity with 10 mmol/l diethyldithiocarbamate, the residual water-soluble superoxide scavenging activity represented less than 25% of total activity and this also showed no difference between control and insulin-treated (1.0 U/day) rats (control 1.84 ± 0.46 and insulin treated 1.80 ± 0.13 IU SOD/mg protein, n = 4 for each group).

To determine the EC-SOD activity, EC-SOD was separated by concanavalin A-sepharose. As expected, aortic tissue showed little EC-SOD activity, and there was no difference in the activity between the two vessels (control 0.66 ± 0.03 and insulin treated 0.68 ± 0.19 U/mg protein, n = 3 for each group). Furthermore, total SOD activities and the other SOD isozymes such as Cu,Zn-SOD and Mn-SOD activities also showed no difference in these two groups [control 11.2 ± 0.58 (10.2 ± 1.1) and insulin treated 9.4 ± 1.3 (8.7 ± 2.1) U/mg protein, n = 3 for each group, Cu,Zn-SOD + Mn-SOD activity was given in parentheses]. To estimate the enzyme activities in endothelial cells, enzyme activities in the absence of endothelium were also
of insulin-treated rats, we also measured O$_2$ production in measuring O$_2$ one previous report discussed a methodological problem in the measurement of enzyme activities was described in MATERIALS AND METHODS. Data are expressed as means ± SE, n = 4. *P < 0.025 and **P < 0.01 vs. control rats.

measured. The activity of EC-SOD and other SOD isozyme activities in endothelium-denuded samples also showed no difference between the two groups (data not shown).

**DISCUSSION**

In the present study, despite the fact that several types of O$_2$-scavenging activities were similar between control and insulin-treated (1.0 U/day) rats, we found endothelial specific increments of O$_2$ production through NAD(P)H oxidases in aortic segments of the insulin-treated rats in both ex vivo and in vitro homogenate conditions. For the measurement of O$_2$ production through activation of NAD(P)H oxidase in ex vivo aortic segments and homogenates, we used the lucigenin-enhanced chemiluminescence method. Although one previous report discussed a methodological problem in measuring O$_2$ using this method (14), several recent reports have confirmed its usefulness (18, 22).

Furthermore, O$_2$ generation released from aortic segments in ex vivo conditions is significantly inhibited in the presence of either NADPH or NADH oxidase inhibitors. To confirm the increased O$_2$ production in vessels of insulin-treated rats, we also measured O$_2$ using the standard cytochrome c method (12) and obtained similar results as found by using the lucigenin chemiluminescence method.

NAD(P)H oxidase activities in vascular homogenate were measured in the presence of either NADPH or NADH in the incubation medium. To evaluate those results, it should be noted that cyclooxygenase-prostaglandin hydperoxidase (PGH synthase) and lipoxygenase can oxidize NAD(P)H and result in the generation of O$_2$ (11). However, a significant increase in O$_2$ production could not be detected when the homogenate was exposed to arachidonic acid (Fig. 2), and neither 10 µmol/l indomethacin nor 10 µmol/l NDGA (an inhibitor of lipoxygenase) affected O$_2$ production in the presence of either NADPH or NADH (data not shown). We noted that neither indomethacin nor NDGA affected vascular O$_2$ production in aortic segments of insulin-treated rats (Table 2). In addition, 1 µmol/l L-NNa (an inhibitor of endothelial NO synthase) did not affect O$_2$ production in both aortic segments and vascular homogenate in insulin-treated rats. These results indicate that O$_2$ production through either arachidonic acid metabolism or endothelial NO synthase activity in ex vivo conditions and in vascular homogenate was not stimulated in insulin-treated hyperinsulinemic rats. Furthermore, the addition of either apocynin (an inhibitor of activity assembly of the components of NADPH oxidase) or diphenyleneiodonium (an inhibitor of flavin-containing enzymes) significantly decreased the lucigenin chemiluminescence in the presence of either NADH or NADPH.

These results also confirmed increased activities of NAD(P)H oxidase in insulin-treated rats. The significance of the membrane-associated NADH oxidoreductase as the main source of O$_2$ has been reported in smooth muscle cells of calf pulmonary artery by Mohazzab and Wolin (18). Thus in the present study we first demonstrated exogenous hyperinsulinemia without insulin resistance specifically activated endothelial NAD(P)H oxidase and then stimulated the generation of O$_2$ in aortas of hyperinsulinemic rats in ex vivo conditions. Interestingly, nonendothelial NAD(P)H oxidase activities in aortic homogenate prepared in the absence of endothelium were not significantly stimulated in insulin-treated rats. These results suggest that insulin differentially regulates vascular NAD(P)H oxidase between the endothelium and smooth muscle cells. In the present study, we were not able to further explain the reason for the tissue specificity of this insulin action.

NADPH oxidase in granulocytes is a multicomponent plasma membrane-bound enzyme that produces O$_2$ in response to stimuli from microbial infections (4). This enzyme consists of transmembrane flavocytochrome b558 (two subunits, gp91phox and p22phox) and regulatory cytosolic proteins, p47phox, p67phox, and small GTPase Rac1 or Rac2, and p40phox (5, 13). Although molecular cloning of this multiple component enzyme has not yet been possible in nongranulocytes, the biological significance of those enzymes in the regulation of vascular function has been recognized (7, 26, 31).

Recently, it has been reported that apocynin, a NADPH oxidase inhibitor is effective in tumor necrosis factor-induced vascular cell adhesion molecule 1 mRNA expression in human umbilical vein endothelial cells (31). Furthermore, p22phox is a critical component of the O$_2$-generating NAD(P)H oxidase system and contributes to angiotensin II-induced hypertrophy of vascular smooth muscle cells (7). However, this NAD(P)H oxidase in smooth muscle cells preferentially uses NADH as opposed to NADPH as a substrate (18, 31).

Molecular mechanisms of this difference need to be studied further. In endothelium, the activation of NAD(P)H oxidase is more likely to be associated with the function of quenching NO or contributing to a direct intracellular...
lar signaling of hormone action that is found in the effects of angiotensin II on smooth muscle cells (26). Concerning the activating mechanism for \( O_2 \) generation in aortic vessels exposed to insulin, we found acute as well as chronic insulin effects on these enzyme activities. However, in contrast to clearly described mechanisms for the activation of NADPH oxidase in granulocytes (4, 13), molecular mechanisms for both acute and chronic activation of NAD(P)H oxidases by insulin in tissues other than granulocytes have not yet been well documented.

We found that the aortas obtained from insulin-treated hyperinsulinemic rats enhanced the production of \( O_2 \) through the activation of endothelial NAD(P)H oxidase. It is also well known that insulin stimulates NO production in endothelial cells. This insulin-stimulated production in both endothelial \( O_2 \) and NO may interact and produce peroxynitrite in vascular tissues. Peroxynitrite at high concentration is cytotoxic and injures various tissues (2). On the other hand, it mediates a number of physiological processes that are beneficial in the protection of cellular function such as coronary arterial relaxation and inhibition of platelet aggregation and leukocyte adhesion (15). Thus peroxynitrite may also be a candidate for modulating vascular function in vivo.

In conclusion, we first showed that physiological hyperinsulinemia could overproduce \( O_2 \) exclusively in endothelial cells. The pathophysiological implication for this endothelial cell-specific activation of NADH-NADPH oxidase by insulin is now being further studied in our laboratory.

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