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# Endothelial- and nitric oxide-dependent effects on oxidative metabolism of intact artery

John T. Barron *, Liping Gu, Joseph E. Parrillo

*Section of Cardiology, Department of Internal Medicine, Rush Medical College, Rush-Presbyterian-St. Luke's Medical Center, 1653 West Congress Parkway, Chicago, IL 60612, USA*

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**Abstract**

Oxidative metabolism and its possible modulation by nitric oxide (NO) was examined in endothelial-intact and endothelial-denuded segments of porcine carotid arteries. Endothelial-intact arteries displayed appropriate NO-mediated vasorelaxation to acetylcholine (ACh). Endothelial-denuded arteries demonstrated absent vasorelaxation to ACh stimulation and depressed contractile responsiveness to $K^+$ depolarization, which was normalized by inhibition of NO synthesis by $N^{\omega}$-nitro-L-arginine methylester (L-NAME). Confirmation that carotid arteries continued to produce NO despite removal of the endothelium was indicated by detection of NO metabolites in the incubation medium bathing the arteries. $O_2$ consumption and the oxidation of glucose and fatty acid were depressed in endothelial-denuded arteries. Depression of $O_2$ consumption and glucose oxidation was completely reversed by treatment with L-NAME. We conclude that endogenous NO produced by non-endothelial vascular cells depresses contractility, $O_2$ consumption, and oxidation of energy substrates in vascular smooth muscle. The endothelium may play a role in oxidative metabolism of vascular smooth muscle possibly by modulating the effects of NO produced by other cells of the vessel wall, or by other factors. 

*Keywords:* Nitric oxide; Vascular smooth muscle; Oxidative metabolism; $O_2$ consumption; Endothelium; Mitochondria

## 1. Introduction

The endothelial layer of the vascular wall is known to exert important regulatory functions over the underlying media muscularis [1]. This regulation is effected predominately by several vasoactive mediators released from the endothelium in a paracine fashion to affect the subjacent cell layers. Nitric oxide (NO) is the best characterized and perhaps most important mediator which is elaborated in the endothelium as well as in other tissue layers of the vessel wall [2]. NO has emerged as an important intercellular and intracellular messenger controlling numerous biochemical, physiological, and cellular processes in the vessel wall. Although the influence of the endothelium and NO on arterial tone, contractile reactivity, and other aspects of vascular smooth muscle physiology has been reasonably well studied, their influence on the energy metabolism of the vessel has not been examined. The purpose of this investigation was to examine oxidative metabolic variables of segments of intact porcine carotid arteries with and without intact endothelia, and to determine what effect NO produced endogenously in the vessel

* Corresponding author. Fax: +1-312-942-5829.
*E-mail address:* jbarron@rush.edu (J.T. Barron).

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wall may exert on smooth muscle metabolism. It was shown that endogenously produced NO depresses oxidative metabolism.

## 2. Materials and methods

Porcine carotid arteries were obtained shortly after killing (approx. 15 min) from a local slaughterhouse. Helical strips were prepared, mounted in organ baths, attached to force transducers for force recordings, and stretched to simulate 100 mmHg mean arterial pressure [3–5]. The arteries were handled in such manner as to preserve the endothelium intact. To remove the endothelium, the endothelial surface was rubbed gently with a forceps. Following this, the arteries were mounted and briefly treated for 30 s with normal incubation medium containing 2 mg/ml of deoxycholate [6,7] after which time the deoxycholate was removed by four rinses with fresh incubation medium. The incubation medium consisted of (in mM) 118 NaCl, 20 $NaHCO_3$, 4.7 KCl, 1.2 $KH_2PO_4$, 1.2 $MgSO_4$, 1.6 $CaCl_2$, and 5.6 glucose at 37°C. It was aerated with a gas mixture of 95% $O_2$/5% $CO_2$. The arterial preparations were pre-incubated in this medium for 1 h, after which time the medium was changed, the passive tension on the arterial strips was readjusted, and the incubation continued for an additional 3 h. In some experiments, 0.3 mM $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) was included in the incubation medium to inhibit nitric oxide synthase, and thus, the production of endogenous nitric oxide [2].

### 2.1. Substrate oxidation

Glucose oxidation and fatty acid (octanoate) oxidation was determined based on the production of $^3H_2O$ from metabolism of [6-$^3$H]glucose (40 μCi/100 ml) and [8-$^3$H]octanoate (100 μCi/100 ml), respectively [4,5]. The $^3H_2O$ present in aliquots of incubation medium was separated from the remaining labeled substrate by using anion exchange column chromatography as previously described [4]. $O_2$ consumption was measured by using a polarographic electrode embedded in a sealed organ bath [3]. After incubation, the arteries were blotted and weighed.

### 2.2. Nitric oxide metabolites

A stable product of NO metabolism is nitrite ($NO_2^-$) which reflects NO production [2,8]. This was measured in aliquots (0.5 ml) of incubation medium using the Greiss reaction [8]. Trace nitrite measured in the incubation medium at time 0 was subtracted from the level measured at 180 min to obtain total nitrite produced over 3 h of incubation.

### 2.3. High-energy phosphates

The content of ATP and phosphocreatine was measured in perchloric acid extracts of arteries. After incubation, the arteries were rapid frozen in liquid $N_2$ and pulverized to a fine powder. Perchloric acid extracts were prepared as previously described and ATP and phosphocreatine were measured using an enzymatic assay [9].

All chemicals and enzymes were purchased from Sigma. [6-$^3$H]glucose was purchased from NEN and [8-$^3$H]octanoate was purchased from American Radiolabeled Chemicals (St. Louis, MO).

### 2.4. Statistics

A Student's *t*-test for paired comparisons was used to assess a change within the same group. When comparing means of three or more groups, one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparisons test was used. Repeated-measures ANOVA followed by the Bonferroni multiple comparisons test was used to assess statistical significance of differences in concentration–response relationships and differences in metabolic rates and other variables between different experimental conditions. Unless noted otherwise, *n* refers to the number of experiments, each with at least two different carotid arteries from different animals. A *P* value of less than 0.05 was significant.

## 3. Results

### 3.1. Vasorelaxation and contractile responsiveness

The functional state of the endothelium and NO-dependent vasodilating responsiveness of porcine

carotid artery strips was assessed by contracting the arteries with a maximal dose of norepinephrine (300 μM, determined by the concentration–response relationship) and then challenging the arterial strips with acetylcholine (ACh), an endothelial and NO-dependent vasodilating agonist [1,2,10]. Table 1 gives the maximal isometric force generated in response to NE and the subsequent change in force upon treatment with acetylcholine. In endothelial-intact arteries which were generating maximal isometric force, introduction of 1 μM acetylcholine to the organ bath produced the expected vasorelaxation and reduction in isometric force (28 ± 4%). Addition of 10 μM ACh did not result in further reduction of force (Table 1). In endothelial-intact arteries which were treated with L-NAME, an inhibitor of nitric oxide synthase and thus NO production, challenge with ACh did not result in relaxation. Rather, isometric force increased by 23 ± 4% at 1 μM ACh. At 10 μM ACh there was an even greater increase in isometric force (56 ± 9%). These results verify the functional integrity of the endothelium of the arterial strip preparation, and the NO-dependency of the vasorelaxation induced by ACh. In endothelial-denuded arteries, 1 μM ACh did not produce vasorelaxation, but vasoconstriction as evidenced by the increase in force (27 ± 4%), thereby verifying the functional removal of the endothelium of the preparation. Increasing the ACh concentration to 10 μM also did not produce vasorelaxation in denuded arteries, and there was no additional increase in isometric force above the level of that at 1 μM ACh.

When L-NAME was included in the incubation medium with endothelial-denuded arteries, there was again no vasorelaxation in response to 1 μM ACh, but vasoconstriction (isometric force increased by 22 ± 4%). In contrast to the case in the absence of L-NAME, increasing the concentration of ACh to 10 μM produced an additional increase in force by 55 ± 12%. This suggested that despite the functional removal of the endothelium and abolition of endothelial-dependent vasorelaxation responsiveness to ACh, NO production of the arteries was not eliminated and the NO produced was affecting the contractile reactivity of the arterial strips.

The hypothesis that endothelial-denuded arteries retained the ability to produce NO was tested by measuring the quantity of a stable product of NO metabolism, $NO_2^-$ (nitrite), present in the incubation medium. At the end of 3 h of incubation, the nitrite produced by endothelial-intact resting arteries was 125 ± 33 nmol/g ($n = 4$). Nitrite was also measurable in the incubation medium bathing endothelial-denuded arteries (88 ± 33 nmol/g, $n = 4$) confirming the assertion that despite de-endothelialization, the preparation continued to produce NO. There was no significant difference in nitrite production in endothelial-intact, when compared to endothelial-denuded arteries. Treatment of arteries with L-NAME significantly inhibited nitrite formation (1 ± 20 nmol/g; $n = 10$, $P < 0.01$).

The effect of removal of the endothelium and the involvement of NO in contractile responsiveness of porcine carotid artery was further assessed by examining the concentration–response relationship in response to contractile stimulation by $K^+$ depolarization (Fig. 1). Treatment of endothelial-intact arteries with L-NAME produced a leftward shift in the concentration–response curve and a significant decrease in the $EC_{50}$ (Table 2). Denudation of the endothe-

Table 1
Effect of removal of endothelium on NO-dependent vasorelaxation

| Condition | NE maximum force | Change in force | |
|---|---|---|---|
| | | ACh, 1 μM | ACh, 10 μM |
| Endothelium-intact | 38 ± 4 | −11 ± 3 | −10 ± 3 |
| Endothelium-denuded | 30 ± 3 | +8 ± 1 | +9 ± 2 |
| Endothelium-intact+L-NAME | 32 ± 3 | +7 ± 1 | +16 ± 3* |
| Endothelium-denuded+L-NAME | 36 ± 4 | +8 ± 2 | +18 ± 3* |

Arteries were contracted with a maximal concentration of norepinephrine (NE) (300 μM). While maintaining isometric force, the arteries were subsequently challenged with acetylcholine (ACh). The change in force from the level during NE contraction was then noted. Values represent mean ± S.E., $n = 16$ arteries from different animals in each condition. *$P < 0.05$ when compared with ACh, 1 μM.

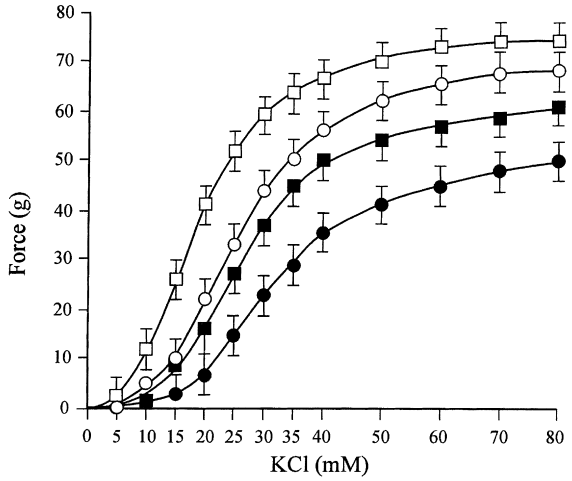


Fig. 1. Concentration–response relationships to $K^+$ depolarization of endothelial-intact and endothelial-denuded arteries. Values represent means ± S.E. (○) Endothelial-intact arteries; (●) endothelial-denuded arteries; (□) endothelial-intact arteries+L-NAME; (■) endothelial-denuded arteries+L-NAME. All concentration–response curves significantly different from one another ($P < 0.05$). $n = 16$ different arteries from different animals in each experimental group.

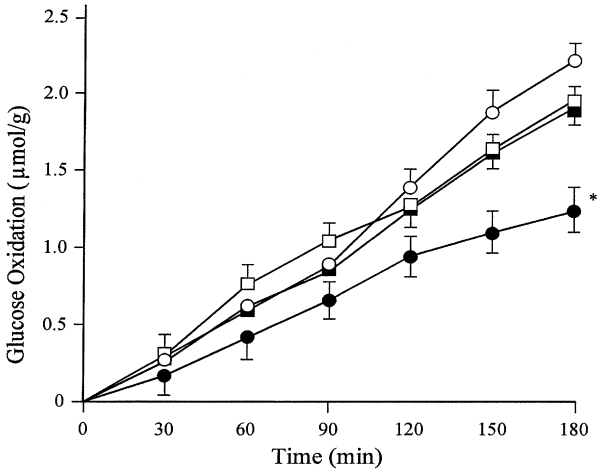


Fig. 2. Glucose oxidation and the effect of NO in endothelial-intact and endothelial-denuded arteries. Values represent means ± S.E. (○) Endothelial-intact arteries; (●) endothelial-denuded arteries; (□) endothelial-intact arteries+L-NAME; (■) endothelial-denuded arteries+L-NAME. $n = 6$ experiments in each group, each with two different carotid arteries from different animals. *$P < 0.0001$.

lium produced a rightward shift in the concentration response relationship and a significant increase in the $EC_{50}$. Subsequent treatment of endothelial-denuded arteries with L-NAME normalized the concentration–response relationship, shifting the curve leftward (Fig. 1 and Table 2).

### 3.2. Oxidative metabolism

After having demonstrated that contractile function of the intact artery is significantly influenced by the endothelium and the NO system, it was of interest to determine their respective roles in the energy metabolism of the arteries. Accordingly, metabolic variables of the endothelial-intact and endothelial-denuded arteries were measured. Fig. 2 shows the time course of the oxidation of glucose of both preparations. In endothelial-intact arteries the oxidation of glucose proceeded at a constant rate with time. In contrast, the rate of glucose oxidation by endothelial-denuded arteries initially remained constant, but after approx. 120 min of incubation in PSS, the rate of glucose oxidation declined. By the end of 3 h, 2.22 ± 0.12 μmol/g glucose had been oxidized by endothelial-intact arteries, whereas, 1.26 ± 0.13 μmol/g glucose had been oxidized by endothelial-denuded arteries ($n = 6$, $P < 0.0005$). The oxidation of fatty acid was also assessed in endothelial-intact and denuded arteries (Fig. 3). The oxidation of the fatty acid octanoate was significantly depressed in endothelial-denuded arteries, (1.10 ± 0.04 μmol/g per 3 h vs. 0.91 ± 0.04 μmol/g per 3 h; $P < 0.013$, $n = 4$) indicating that removal of the endothelium depressed

Table 2
Contractile responsiveness of carotid arteries to $K^+$ depolarization

| Condition | $EC_{50}$ (mM) | Maximal force (g) |
|---|---|---|
| Endothelial-intact (control) | 25.9 ± 1.5 | 68.1 ± 4.2 |
| Endothelial-denuded | 32.9 ± 1.5* | 50.0 ± 4.2* |
| Endothelial-intact+L-NAME | 20.6 ± 1.5* | 74.8 ± 4.2 |
| Endothelial-denuded+L-NAME | 26.6 ± 1.5 | 61.0 ± 4.2 |

Values represent means ± S.E., $n = 16$ different carotid arteries from different animals in each experimental condition. *$P < 0.05$ when compared with control.

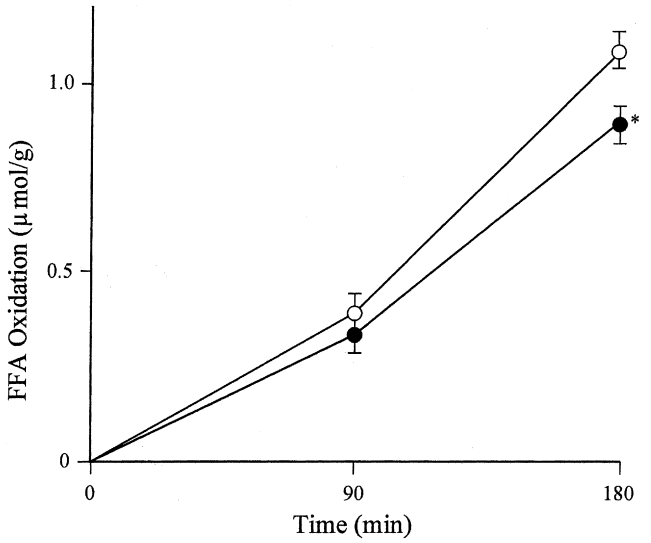


Fig. 3. Free fatty acid oxidation in endothelial-intact and endothelial-denuded arteries. Values represent means ± S.E. (○) Endothelial-intact arteries; (●) endothelial-denuded arteries. $n = 4$ experiments in each group, each with two different carotid arteries from different animals. $^*P < 0.013$.

oxidative metabolism of the tissue. The involvement of NO in depression of oxidation of substrates was assessed by measuring the oxidation of glucose in endothelium-denuded arteries in the presence of L-NAME (Fig. 2). Glucose oxidation by denuded arteries under these conditions was no different from control, i.e., L-NAME negated the effect of removal of the endothelium on oxidative metabolism (Fig. 2). L-NAME had no effect on glucose oxidation in endothelial-intact arteries.

Further evidence that the endothelium and NO play a role in the oxidative metabolism of arteries was obtained from measurement of $O_2$ consumption in endothelial-intact and endothelial-denuded arteries. Oxygen consumption in endothelium-denuded arteries ($0.41 \pm 0.02$ μmol/g per min, $n = 13$) was significantly less than that of endothelial-intact arteries ($0.55 \pm 0.04$ μmol/g per min, $n = 11$, $P < 0.01$). There was no difference between the blotted wet weights of endothelial-intact arteries and endothelial-denuded arteries used for $O_2$ consumption measurements ($0.441 \pm 0.011$ g, $n = 25$) vs. ($0.445 \pm 0.013$ g, $n = 25$; $P =$ not significant (NS)). L-NAME completely normalized $O_2$ consumption in endothelial-denuded arteries, suggesting that NO was involved in depression of $O_2$ consumption of vascular smooth muscle (Fig. 4).

The fact that L-NAME reversed the depression of oxidative metabolism in endothelial-denuded arteries indicates that such depression could not be due to loss of the portion of total tissue respiration contributed by the endothelium. Also, the depression in oxidative metabolism was not due to a change in the energy requirements of the muscles since there was no difference in resting tone of the arteries in any experimental condition (data not shown). Furthermore, there was no difference in high energy phosphate content (ATP+phosphocreatine) in endothelial-intact and endothelial-denuded arteries ($2.38 \pm 0.05$ μmol/g vs. $2.34 \pm 0.13$ μmol/g; $n = 8$, $P =$ NS).

When comparing $O_2$ consumption of endothelial-intact arteries in the presence or absence of L-NAME (Fig. 4), arteries treated with L-NAME displayed a $9.3 \pm 2.9\%$ increase in $O_2$ consumption from the basal rate before treatment for 3 h with L-NAME ($n = 11$, $P < 0.005$; $t$-test of paired comparisons). By contrast, $O_2$ consumption of control, endothelial-intact arteries not treated with L-NAME, did not

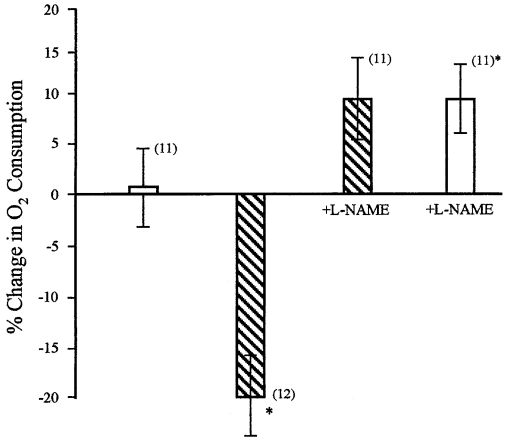


Fig. 4. $O_2$ consumption and the effect of NO in endothelial-intact and endothelial-denuded arteries. Basal $O_2$ consumption was measured after 1 h incubation in normal medium. Subsequently, either the endothelial layer was removed and/or the incubation medium was changed to the one indicated. $O_2$ consumption was again measured 3 h later and the change from the pre-treatment rate was noted. Values represent means ± S.E. and are expressed in terms of percent change from the pre-treatment rate in the same arteries. Open bars, endothelial-intact arteries; hatched bars, endothelial-denuded arteries. Numbers in parentheses ($n$) indicate number of experiments each with two different arteries from different animals. $^*P < 0.05$, when compared to endothelial-intact arteries, in the absence of L-NAME (control).

change from the basal rate after additional incubation in normal medium ($0.5 \pm 1.8\%$; $n = 11$, $P = \text{NS}$). The increase in $O_2$ consumption in the presence of L-NAME was significantly increased when compared to control. These results suggest that endogenous NO produced from cells in the vessel wall did have a small but detectable inhibitory effect on tissue respiration even in endothelial-intact arteries.

## 4. Discussion

### 4.1. Endothelium-dependent effects

The role of the endothelium in the energy metabolism of arteries has received little investigation. One early report indicated that $O_2$ consumption and glucose metabolism in isolated segments of rat aorta were significantly different if the segments were prepared in such a way as to preserve the endothelium intact, as compared to when the endothelium was disrupted [11]. Rat aortae with intact endothelia exhibited a greater rate of $O_2$ consumption and glucose oxidation than aortae with disrupted endothelia. The results with porcine carotid arteries in the present investigation are in agreement with this previous report. We also demonstrated that fatty acid oxidation was also significantly decreased when the endothelial layer was removed, indicating that the effect of endothelial denudation was not specific to glucose metabolism. Rather, the results showing depression of glucose metabolism, fatty acid oxidation, and $O_2$ consumption suggest that the endothelial denudation had a general affect on oxidative metabolism.

Removal of the endothelial layer, in addition to depressing oxidative metabolism, also depressed contractile responsiveness. There was a rightward shift in the concentration–response relationship to $K^+$-depolarization, a significant increase in $EC_{50}$ to KCl, and a significant decrease in maximal force generated. The endothelium is known to secrete several vasoactive mediators that may either enhance or depress resting tone and/or contractility of the underlying media muscularis [1,12]. The summed effect of the various vasoactive factors on contractile reactivity would depend on the physiological milieu and the activating stimuli. Conversely, withdrawal of these mediators would also variably affect contractile responsiveness. In porcine carotid artery, the net effect of removal of endothelium was apparently depression of contractile responsiveness of the smooth muscle. By contrast, resting tone was apparently unaffected since there was no difference in the resting tension between the endothelial-intact and endothelial-denuded preparations (data not shown).

### 4.2. Nitric oxide-dependent effects

The experimental evidence indicated that NO was responsible for the depression in $O_2$ consumption and oxidative metabolism in endothelial-denuded arteries. Depression of $O_2$ consumption was eliminated by L-NAME. Similarly, L-NAME normalized glucose oxidation and $O_2$ consumption in endothelial-denuded arteries. The depression of metabolism in vascular smooth muscle by NO is consistent with other reports of its metabolic action in other tissues [13–16]. NO has been reported to suppress $O_2$ consumption and mitochondrial respiration in cardiac muscle and other tissues by competing with molecular $O_2$ at the mitochondrial electron transport chain. Specifically, cytochrome *c* oxidase activity may be competitively inhibited by NO [13,18,19]. NO has also been demonstrated to govern the proton gradient, and hence, the electrical potential across the inner mitochondrial membrane. Ghafourihar and Richter [17] demonstrated that exogenously added NO caused a dose-dependent acidification of the inner mitochondrial matrix resulting in a drop in the mitochondrial transmembrane potential. Activation of mitochondrial ATP-dependent $K^+$ channels is another reported action of NO which could affect mitochondrial function [20]. These actions of NO could have important consequences on oxidative metabolism of the tissue. Dissipation of the mitochondrial transmembrane potential and direct inhibition of $O_2$ consumption could adversely affect the oxidation of substrates. In contrast to these studies, other studies with skeletal muscle report that NO stimulates oxidative metabolism by a cGMP-mediated mechanism involving activation of cGMP-mediated protein kinase (PKG) [21–23]. The authors of the studies speculated that activation of PKG and attendant stimulation of oxidation of metabolic fuels may supersede any inhibiting effect of NO on respiration. Thus, NO may have multiple counterbalancing ef-

fects on cellular energy producing pathways. In our study with vascular smooth muscle, the prevailing effect of NO appears to be suppression of $O_2$ consumption and oxidation of metabolic fuels.

The evidence indicated that the depression of contractile reactivity to $K^+$ depolarization was also in large part due to NO. Treatment of the de-endothelialized arterial strips with L-NAME improved contractile reactivity. NO is known to depress contractile responsiveness of vascular smooth muscle [24,25], and was probably responsible for the rightward shift in the concentration–response relationship (relative to the concentration–response relationship in the presence of L-NAME) in endothelial-denuded preparations, and also in endothelial-intact preparations. However, the concentration–response relationship in endothelial-denuded arteries did not completely normalize upon treatment with L-NAME and it was still shifted rightward relative to endothelial-intact arteries which were also treated with L-NAME. Therefore, the depression of contractile responsiveness in endothelial-denuded arteries was greater then what could be completely attributed to NO alone. Rather, removal of the endothelium may have concomitantly resulted in withdrawal of other endothelial-derived factors which have vasoconstrictive properties (e.g., endothelin) and which might have counterbalanced the vasodepressive action of NO.

### 4.3. Extra-endothelial source of NO

Quiescent, resting porcine vascular endothelial cells in culture produce little or no ambient NO which can be detected in the incubation medium unless the cells are activated by physiological stimuli such as stretch or vasoactive agents which increase the influx of $Ca^{2+}$ [26]. $Ca^{2+}$ influx is apparently crucial for NO generation by nitric oxide synthase in endothelial cells both under basal conditions and upon stimulation by pharmacological agonists, such as acetylcholine [27–30]. Because NO production (as reflected by total nitrite production) was not changed when the endothelium was removed, it is assumed that the majority of ambient NO production in the intact resting artery originated not from the endothelium, but from other cells in the tunica media muscularis or tunica adventitia. Several cells of the vessel wall including macrophages, fibroblasts and vascular smooth muscle cells have the ability to elaborate NO and other vasoactive substances such as superoxide in cell culture [2,31]. In fact, fibroblasts from adventitia were demonstrated to be a major source of NO and superoxide production in intact rat or mouse aortae [31,32]. Nevertheless, the ambient NO production from cells in the vessel wall other than endothelial cells might also be expected to depress oxidative metabolism in endothelial-intact arteries, as occurred in endothelial-denuded arteries. Indeed, there was a small but significant (approx. 9%) increase in $O_2$ consumption in endothelial-intact arteries when treated with L-NAME and L-NAME did produce a leftward shift in the concentration response relationship to KCl. However, an effect on glucose oxidation was not detected most likely because any increase would be small and would be difficult to detect due to the limitation of the analytical methods for determination of glucose oxidation. The relatively small effect of endogenous NO production on endothelial-intact arteries, in comparison to endothelial-denuded arteries, may be due to multiple factors. It may be the case that higher concentrations of NO are needed to affect the energy metabolism of vascular smooth muscle. In this context it is worth noting that recent evidence suggests that the concentration of bioactive NO is decreased in muscle cells because of the presence of myoglobin. Myoglobin acts essentially as a scavenger of bioactive NO [33,34]. In an analogous manner, the endothelium may have exerted an effect in endothelium-intact arteries to counteract the metabolic actions of NO produced from the subjacent vessel wall layers by inactivating NO. Incomplete removal of the endothelium and differential sensitivity to the inhibitory action of NO of different cell types in the vessel wall may also account for the experimental observations that L-NAME reversed the depression of $O_2$ consumption in 'endothelial-denuded' arteries. Alternatively, the endothelium may have counteracted the inhibitory metabolic effects of NO by elaborating some endothelial-derived 'metabolism regulating factor'.

In summary, our results show that the removal or disruption of vascular endothelial layer results in depression in oxidative metabolism and contractility of carotid artery. It was also shown that such depression was attributed to NO which likely originated from cells within the tunica media muscularis and/

or adventitia. The endothelium modulates oxidative metabolism of the vessel wall possibly by modulating the effects of NO and perhaps other substances produced by other cells, or by other factors. The regulation of the production of NO in extra-endothelial cells in the intact artery is not defined at present.

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