Brazilian Journal of Medical and Biological Research (2004) 37: 1215-1224
ISSN 0100-879X

Protective effect of N-acetylcysteine against oxygen radical-mediated coronary artery injury

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Abstract

The present study investigated the protective effect of N-acetylcysteine (NAC) against oxygen radical-mediated coronary artery injury. Vascular contraction and relaxation were determined in canine coronary arteries immersed in Kreb's solution (95% O_2-5% CO_2), incubated or not with NAC (10 mM), and exposed to free radicals (FR) generated by xanthine oxidase (100 mU/ml) plus xanthine (0.1 mM). Rings not exposed to FR or NAC were used as controls. The arteries were contracted with 2.5 µM prostaglandin F2α. Subsequently, concentration-response curves for acetylcholine, calcium ionophore and sodium fluoride were obtained in the presence of 20 µM indomethacin. Concentration-response curves for bradykinin, calcium ionophore, sodium nitroprusside, and pinacidil were obtained in the presence of indomethacin plus N^{ω}-nitro-L-arginine (0.2 mM). The oxidative stress reduced the vascular contraction of arteries not exposed to NAC (3.93 ± 3.42 g), compared to control (8.56 ± 3.16 g) and to NAC group (9.07 ± 4.0 g). Additionally, in arteries not exposed to NAC the endothelium-dependent nitric oxide (NO)-dependent relaxation promoted by acetylcholine (1 nM to 10 µM) was also reduced (maximal relaxation of 52.1 ± 43.2%), compared to control (100%) and NAC group (97.0 ± 4.3%), as well as the NO/cyclooxygenase-independent receptor-dependent relaxation provoked by bradykinin (1 nM to 10 µM; maximal relaxation of 20.0 ± 21.2%), compared to control (100%) and NAC group (70.8 ± 20.0%). The endothelium-independent relaxation elicited by sodium nitroprusside (1 nM to 1 µM) and pinacidil (1 nM to 10 µM) was not affected. In conclusion, the vascular dysfunction caused by the oxidative stress, expressed as reduction of the endothelium-dependent relaxation and of the vascular smooth muscle contraction, was prevented by NAC.

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Rersearch supported by the Mayo Foundation, Rochester, NY, USA and in part by FAPESP.

Received March 26, 2003
Accepted June 8, 2004

Key words
• Coronary arteries
• N-acetylcysteine
• Endothelium
• Oxidative stress
• Nitric oxide

Introduction

The vascular endothelium is intimately involved in the pathophysiology of ischemia-reperfusion injury. Consequently, strategies to avoid or minimize the resulting endothelial dysfunction after ischemia-reperfusion have gained interest, and among them the use of antioxidants is promising (1).

N-acetylcysteine (NAC) has been used

for almost half a century to treat congestive and obstructive lung diseases, and since the mid-1970's it has also been used to treat paracetamol intoxication (2). Several recent reports have suggested that its use may be extended to manage clinical conditions as diversified as ischemia-reperfusion injury (3), inflammation-related complications after cardiopulmonary bypass (4) and renal damage provoked by radiographic contrast (5).

As an extensively used and relatively safe drug (6), NAC may be a useful adjuvant for the protection of the myocardium in cardiac surgery, for example as an additive to the cardioplegic solutions. Therefore, given the role of the vascular endothelium in the control of vascular function and its ability to attenuate ischemia-reperfusion-related injuries to the myocardium (7), the present study was carried out in order to determine the effects of oxidative stress on the vascular reactivity of canine coronary arteries, as well as the protective role of NAC in an *in vitro* model of oxidative stress (8).

Material and Methods

Animal preparation

Heartworm-free mongrel dogs (25-30 kg) of either sex were anesthetized with intravenous sodium thiopental (30 mg/kg; Abbott Labortatories, Chicago, IL, USA) and exsanguinated by cutting the carotid arteries. The chest was quickly opened and the beating heart was harvested and immersed in cool oxygenated physiologic Kreb's solution of the following composition: 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.22 mM KH_2PO_4, 2.5 mM $CaCl_2$, 25.0 mM $NaHCO_3$, and 11.1 mM glucose. The procedures and handling of the animals were in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and in agreement with the Institutional Animal Care and Use Committee of the Mayo Foundation.

In vitro experiments

The left circumflex coronary artery was carefully dissected and vascular rings (4-5 mm in length) were obtained. The vascular rings were suspended in organ chambers (25 ml) filled with oxygenated Kreb's solution maintained at 37ºC and bubbled with 95% O_2-5% CO_2, pH 7.4. Each ring was suspended on two stainless steel clips passed through its lumen. One clip was anchored to the bottom of the organ chamber, and the other was connected to a strain-gauge for measurement of isometric force (Statham UC 2, Gould, Cleveland, OH, USA). After stabilization, they were progressively stretched to the length-tension of 10 g. In order to ensure that the endothelium of the rings was not damaged during handling, the rings were contracted with 20 mM KCl. After reaching a plateau of contraction, the relaxing response to 1 μM acetylcholine was tested. The endothelium was considered to be functional when the vessel was relaxed by at least 90% (considering the plateau of contraction as 0% relaxation). Subsequently, the rings were allowed to equilibrate in Kreb's solution without any drug for a period of 30 min before the addition of any drug.

Experimental design

The experiments were carried out with sets of three rings for each drug tested. One ring was not exposed to the free radicals or NAC (control) and one ring was incubated with 10 mM NAC for 60 min before the oxidative stress (NAC group). NAC was maintained in the bath during exposure to the oxygen-derived free radicals. The third ring was not exposed to NAC before or during exposure to the oxygen-derived free radicals (non-NAC group).

Exposure to oxygen-derived free radicals

Xanthine oxidoreductase is a molybdoenzyme capable of catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism. It reduces NAD^+ and leads to the production of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (8). Oxygen-derived free radicals were generated by incubating all vascular rings, except the controls, for 90 min in oxygenated Kreb's solution containing xanthine oxidase (100 mU/ml) and 0.1 mM xanthine. The vascular rings were then washed four times with Kreb's solution without any drug and allowed to stabilize for 30 min before drug addition.

Vascular contraction study. Contraction was stimulated with 2.5 µM prostaglandin F2α and the relaxation study was started after the vessel reached a stable tonic contraction.

Vascular relaxation study. In order to assess the effects of oxidative stress on the endothelium-dependent relaxation promoted by nitric oxide (NO-dependent relaxation), all experiments were carried out by blocking the synthesis of relaxing prostaglandins (cyclooxygenase-dependent relaxing factors), mainly prostacyclin I_2, by adding 20 µM indomethacin, a cyclooxygenase inhibitor, to the organ chambers.

We also determined the effects of the oxidative stress on the endothelium-dependent relaxation not related to the release of NO and relaxing prostaglandins (NO- and cyclooxygenase-independent relaxation) caused by the hypothesized endothelium-derived hyperpolarizing factor (EDHF) by adding to the organ chambers the NO synthase (NOS) inhibitor 0.2 mM N^ω-nitro-L-arginine in addition to indomethacin.

All enzyme blockers were added to the chamber at least 45 min before starting the determination of the concentration-response curves. Rings that did not present a plateau of contraction of at least 1 g of tension were excluded from the relaxation study.

Endothelium-dependent relaxation

Receptor-dependent NO-dependent relaxation. In order to study the receptor-dependent NO-dependent relaxation the rings were exposed to increasing concentrations of acetylcholine (1 nM to 10 µM).

Receptor-independent NO-dependent relaxation. In order to appraise the receptor-independent NO-dependent relaxation, concentration-response curves for calcium ionophore (1 nM to 10 µM) and sodium fluoride (0.5 to 9.5 M) were constructed. Sodium fluoride, a drug that activates G-proteins directly (9), was used in order to determine if the impairment of G-proteins may participate in receptor-dependent impairment of the production and release of relaxing factors by the endothelium.

Calcium ionophore increases the sarcoplasmic concentration of Ca^{2+} without stimulating a membrane receptor, resulting in the activation of NOS and the production of NO (10).

Receptor-dependent NO- and cyclooxygenase-independent relaxation. In order to study the receptor-dependent NO- and cyclooxygenase-independent relaxation, N^ω-nitro-L-arginine (0.2 mM), an NOS blocker, was used in addition to indomethacin. After reaching the plateau of contraction the rings were exposed to increasing concentrations of bradykinin (1 nM to 10 µM). In the presence of indomethacin and N^ω-nitro-L-arginine the stimulation of the bradykinin receptors promotes the release of EDHF (11).

Receptor-independent NO- and cyclooxygenase-independent relaxation. In the presence of indomethacin and N^ω-nitro-L-arginine, the calcium ionophore also promotes the release of EDHF independent of the NO or cyclooxygenase pathways. Therefore, the vessels were exposed to increasing concentrations of calcium ionophore (1 nM to 10 µM) in the presence of 0.2 mM N^ω-nitro-L-

arginine in addition to 20 µM indomethacin.

Endothelium-independent relaxation. Two different mechanisms of endothelium-independent relaxation were studied, the cyclic GMP (cGMP)-dependent pathway and smooth muscle hyperpolarization. The rings were incubated with 20 µM indomethacin plus 0.2 mM N^{ω}-nitro-L-arginine before contraction with 2.5 µM prostaglandin F2α. After reaching the plateau of contraction, concentration-response curves for sodium nitroprusside (1 nM to 1 µM) and pinacidil (1 nM to 10 µM) were constructed.

Sodium nitroprusside is metabolized by blood vessels to its active metabolite, NO, independently of the presence of a functioning endothelium. Therefore, the resulting relaxation is a consequence of the formation of cGMP in vascular smooth muscle (10).

Pinacidil directly activates ATP-sensitive potassium channels, resulting in smooth muscle hyperpolarization. The hyperpolarization closes voltage-dependent Ca^{2+} channels, resulting in the reduction of intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration and vasodilation (10).

Drugs

The following drugs were used: acetylcholine, bradykinin, indomethacin, N^{ω}-nitro-L-arginine, prostaglandin F2α, sodium nitroprusside, sodium fluoride, xanthine, pinacidil, DMSO, and calcium ionophore, all from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Xanthine oxidase was from Calbiochem (San Diego, CA, USA). All drugs were prepared with distilled water, except indomethacin which was dissolved in 5 M $NaHCO_3$ in distilled water, and pinacidil that was dissolved in DMSO according to the manufacturer instructions.

Statistical analysis

Data are reported as means ± SD. Vascular relaxation is reported as the percentage of the plateau of contraction, with the plateau of contraction being 0% relaxation. Data were analyzed statistically by ANOVA, two-way analysis of variance and Bonferroni post-tests (GraphPad Prism 3.0 Software), with the level of significance set at $P < 0.05$.

Results

Vascular contraction

Oxidative stress provoked a significant impairment of vascular contraction in non-NAC coronary arteries (3.93 ± 3.42 g, N = 42), compared to the control and NAC groups ($P < 0.001$, Figure 1). However, there was no significant difference between the plateau of contraction of rings incubated with NAC (9.07 ± 4.0 g, N = 44) and control rings (8.56 ± 3.16 g, N = 44). In two sets of experiments the rings from the non-NAC group presented no contraction at all and therefore were excluded from the vascular relaxation study.

Vascular relaxation study

Endothelium-dependent relaxation

Receptor-dependent NO-dependent relaxation. The receptor-dependent NO-dependent relaxation (Figure 2) promoted by acetylcholine was significantly impaired in non-NAC (N = 7) coronary arteries. There was no significant difference between the concentration-response curves of the control group (N = 7) and the NAC group (N = 7).

Receptor-independent NO-dependent relaxation. A significant reduction of the receptor-independent NO-dependent relaxation provoked by sodium fluoride (N = 6) was observed in the non-NAC group (Figure 3A). Even though the concentration-response curve for the NAC group (N = 7) presented a shift to the right, the difference was significant only for the concentration of 5.5 M compared with control arteries (N = 7). The maximal receptor-independent NO-depend-

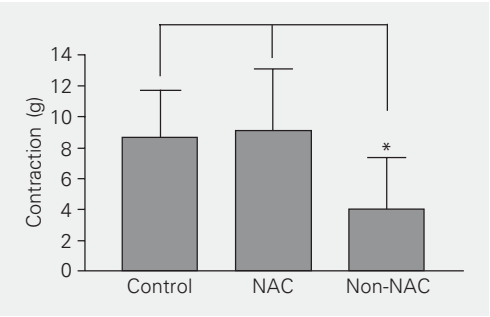

Figure 1. Contraction of canine coronary arteries of the control (N = 44), N-acetylcysteine (NAC, N = 44) and non-NAC (N = 42) groups stimulated with 2.5 µM prostaglandin F2α. The concentration of NAC in the bath was 10 mM. Data are reported as means ± SD. *P < 0.001 for the non-NAC group compared to the control and NAC groups (ANOVA and Bonferroni's multiple comparison test). There was no difference between control and NAC.

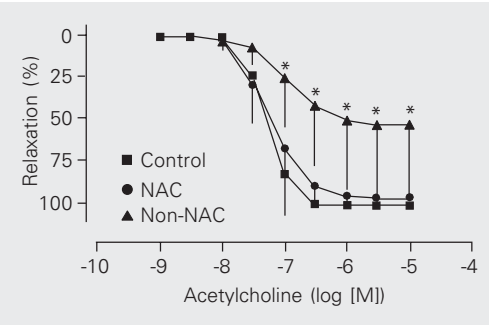

Figure 2. Concentration-response curves for acetylcholine (receptor-dependent NO-dependent relaxation) of canine coronary arteries from the control (N = 7), N-acetylcysteine (NAC, N = 7) and non-NAC (N = 7) groups. The coronaries were incubated with 20 µM indomethacin and contracted with 2.5 µM prostaglandin F2α. Data are reported as means ± SD. *P < 0.001 for the non-NAC group compared to control and P < 0.01 for the non-NAC group compared to the NAC group (two-way analysis of variance and Bonferroni post-tests).

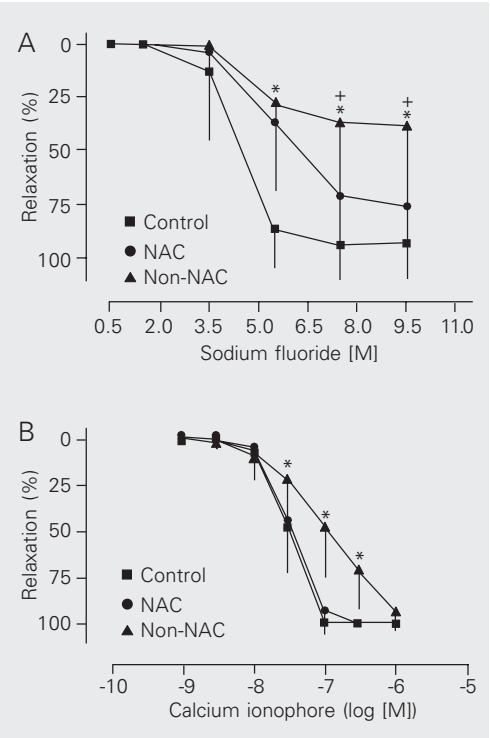

Figure 3. Concentration-response curves for sodium fluoride (A; receptor-independent NO-dependent relaxation) and for calcium ionophore (B; receptor-independent NO-dependent relaxation) of canine coronary arteries from the control (N = 7), (NAC, N = 7) and non-NAC (N = 6) groups. The coronaries were incubated with 20 µM indomethacin and contracted with 2.5 M prostaglandin F2α. Data are reported as means ± SD. (A) $^{+}$P < 0.05 for the non-NAC group compared to the NAC group; *P < 0.001 for the non-NAC group compared to control. (B) *P < 0.001 for the non-NAC group compared to the NAC and control groups (two-way analysis of variance and Bonferroni post-tests).

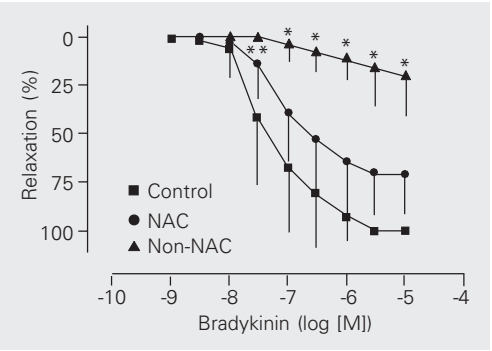

Figure 4. Concentration-response curves for bradykinin (receptor-dependent NO- and cyclooxygenase-independent relaxation) on coronary arteries from dogs of the control (N = 6), N-acetylcysteine (NAC, N = 6) and non-NAC (N = 6) groups. The coronaries were incubated with 20 µM indomethacin plus 0.2 mM N^{ω}-nitro-L-arginine and contracted with 2.5 µM prostaglandin F2α. Data are reported as means ± SD. *P < 0.001 for the non-NAC group compared to the control and NAC groups (two-way analysis of variance and Bonferroni post-tests).

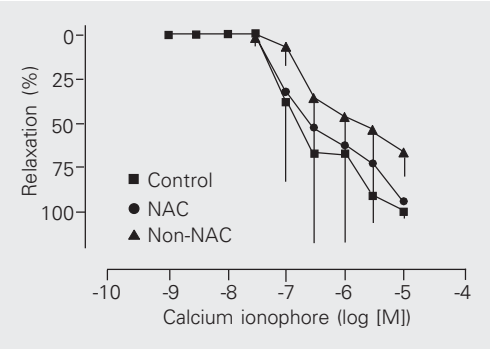

Figure 5. Concentration-response curves for calcium ionophore (receptor-independent NO- and cyclooxygenase-independent relaxation) on coronary arteries from dogs of the control (N = 6), N-acetylcysteine (NAC, N = 6) and non-NAC (N = 6) groups. The coronaries were incubated with 20 µM indomethacin plus 0.2 mM N^{ω}-nitro-L-arginine (receptor-independent NO-independent) and contracted with 2.5 µM prostaglandin F2α. Data are reported as means ± SD. There were no statistical differences in the concentration-response curves of the three groups (two-way analysis of variance and Bonferroni post-tests).

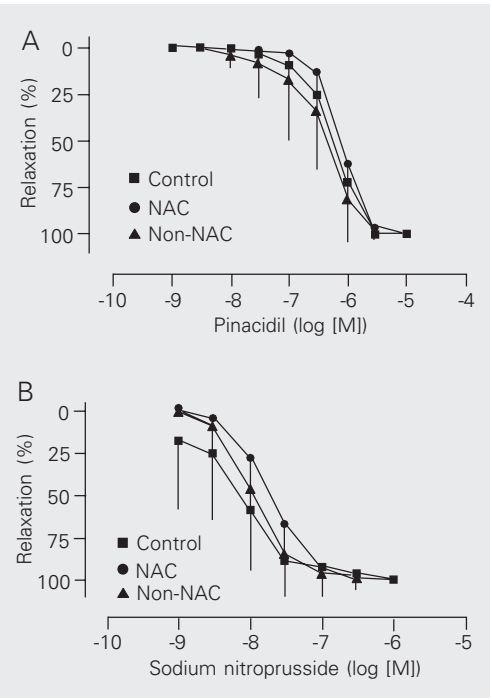

Figure 6. Concentration-response curves for pinacidil (A; N = 5 for each group) and sodium nitroprusside (B; N = 6 for each group) on coronary arteries from dogs of the control, N-acetylcysteine (NAC) and non-NAC groups (endothelium-independent relaxation). The coronaries were incubated with 20 µM indomethacin plus 0.2 mM N^{ω}-nitro-L-arginine and contracted with 2.5 µM prostaglandin F2α. Data are reported as means ± SD. There was no difference between groups in the concentration-response curves (two-way analysis of variance and Bonferroni post-tests).

ent relaxation elicited by calcium ionophore (Figure 3B) was not significantly affected by oxidative stress, although a significant right shift of the concentration-response curve for intermediate concentrations (30 to 300 nM) was observed.

Receptor-dependent NO- and cyclooxygenase-independent relaxation. The receptor-dependent NO- and cyclooxygenase-independent relaxation induced by bradykinin (Figure 4) was significantly impaired in coronary arteries of the non-NAC group (N = 6). There was no significant difference between the concentration-response curves of the control group (N = 6) and the NAC group (N = 6).

Receptor-independent NO- and cyclooxygenase-independent relaxation. The receptor-independent NO- and cyclooxygenase-independent relaxation provoked by calcium ionophore (N = 6 for each group; Figure 5) was not affected by oxidative stress since there was no difference between the concentration-response curves of the three groups of coronary arteries.

Endothelium-independent relaxation

Figure 6 shows the endothelium-independent relaxation elicited by sodium nitroprusside (N = 6 for each group) and pinacidil (N = 5 for each group). There was no impairment of the endothelium-independent relaxation elicited by the activation of the ATP-sensitive potassium channels (pinacidil; Figure 6A) and by the cGMP-dependent pathway (sodium nitroprusside; Figure 6B).

Discussion

The data presented here demonstrate that oxidative stress impaired not only the arterial contraction, but also endothelium-dependent relaxation, mainly the NO-dependent relaxation. It is also evident that NAC prevented the injury promoted by oxidative stress.

The reduction of endothelium-dependent relaxation (8,12-14) and of vascular smooth muscle contraction (15,16) caused by oxidative stress is well known. There are several sources of reactive oxygen species in vascular cells, three of which have been extensively studied (12): a) xanthine oxidase, b) NADH/NADPH oxidase, and c) endothelial NOS (eNOS). As already observed, xanthine oxidase leads to the production of O_2^- and H_2O_2. Ischemia and hypoxia are situations in which the activity of xanthine oxidase is increased. O_2^- reacts with NO to form peroxynitrite, a less effective activator of guanylyl cyclase, resulting in a marked reduction in NO bioactivity (17). Another important source of O_2^- in many cardiovascular diseases is an NADH/NADPH oxidase (12). Finally, a third source of interest is eNOS. There is evidence that in the absence of either L-arginine or tetrahydrobiopterin, eNOS can produce O_2^- and H_2O_2, a phenomenon called NOS uncoupling, whose mechanism is not completely understood (12). Therefore, NOS uncoupling may result in endothelial dysfunction by reducing the production of NO and contributing to the oxidative stress.

The direct effect of free radicals on the endothelial cells is not the unique factor responsible for the reduction of endothelial function. Oxidized low-density lipoproteins are cytotoxic to endothelial cells (18) and there is evidence that oxidized low-density lipoprotein and products of lipid peroxidation can react directly with NO and abolish its biological activity (19). In addition, lipid peroxidation may interfere with signal transduction and receptor-dependent stimulation of NOS activity (20) and with the activation of guanylyl cyclase (21).

The results obtained here with sodium nitroprusside and pinacidil rule out the explanation that the incapacity of the vascular smooth muscle to relax is due to a dysfunction of the cGMP pathway or to the impairment of the hyperpolarizing mechanisms of

smooth muscle. Since receptor-dependent relaxation elicited by acetylcholine and receptor-independent NO-dependent relaxation provoked by calcium ionophore and sodium fluoride were equally affected, we must consider that oxidative stress impaired the NO-dependent relaxation probably by disturbing several steps of the NO pathway.

In addition to the mechanisms mentioned above, dysfunction of other cellular processes may contribute not only to the impairment of the endothelium-dependent vasodilation, but also to the impairment of the vascular smooth muscle contraction. It has been demonstrated that receptor-stimulated Ca^{2+} influx into endothelial cells is biphasic. There is an initial increase in $[Ca^{2+}]_i$ provided by the endoplasmic reticulum, followed by a second sustained elevation of $[Ca^{2+}]_i$ due to a Ca^{2+} influx from an extracellular source (22). Impairment of agonist-stimulated Ca^{2+} signaling pathways provoked by peroxides has been demonstrated in endothelial cells (23). It seems that such impairment is due to the inhibition of the mechanisms responsible for intracellular Ca^{2+} release and extracellular Ca^{2+} influx (23). Consequently, disorders of Ca^{2+} homeostasis provoked by oxidative stress may impair the Ca^{2+} signaling pathway necessary to produce and release endothelium-dependent relaxing factor and to contract smooth muscle (16,24-26). In addition, Na^+-pump dysfunction caused by oxidative stress has been described as a cause of smooth muscle dysfunction as well (27,28).

Dysfunction of G-proteins is another mechanism that may also have contributed to the impaired receptor-stimulated activation of the pathways leading to the production and release of endothelium-dependent relaxing factor, as suggested by our results with sodium fluoride and by previous investigations (8,13,14).

As is the case for NO, the release of NO- and cyclooxygenase-independent endothelium-dependent relaxing factor by coronary arteries also relies on a Ca^{2+} signaling-dependent pathway. There is evidence suggesting that in the coronary arteries of a variety of species such factor may be an epoxyeicosatrienoic acid (29) or even a hydroxyeicosatetraenoic acid (30), both produced by enzymatic pathways that share with the cyclooxygenase pathway the enzyme phospholipase A2, a known calmodulin-dependent enzyme (11). In the present experiments we observed that only the receptor-dependent NO- and cyclooxygenase-independent relaxation evoked by bradykinin was significantly impaired, although our results do not allow us to propose a detailed mechanism.

Few studies have focused on the effects of oxidative stress on EDHF, but EDHF-mediated vascular relaxation resistance to oxidative stress has been described (31-33). The precise mechanism by which the EDHF-mediated vascular relaxation may resist to the deleterious effects of oxidative stress is not known, but up-regulation of EDHF production, as a consequence of NOS inhibition or reduction of NO bioactivity, and less interference of oxidative stress with the EDHF mechanisms of relaxation have been suggested (31-33).

As addressed before, there are compelling data suggesting that oxidative stress is an important trigger of the complex events leading to endothelial dysfunction after ischemia and in other cardiovascular diseases. Therefore, the use of drugs to prevent oxygen radical-mediated injury has been extensively investigated. NAC, whose the antioxidant properties are well know (2,34,35), has been extensively investigated for decades as an adjuvant for the treatment of cardiovascular disorders (35-39). Because of its SH group, NAC has the potential to interact directly with oxidants such as H_2O_2 to form H_2O and O_2 (34), and like many thiols, such as reduced glutathione (GSH), is a hydroxy radical scavenger (40). In addition, thiol NAC is readily deacetylated in the cells to yield L-cysteine, thereby promoting intracellular

GSH synthesis (35), one of the most important cellular defenses against oxidative stress. It has also been proposed that NAC may improve endothelial function, enhancing the bioavailability of NO by spontaneously forming nitroso-N-acetylcysteine and S-nitrosocysteine that could act as diffusion-limited NO adducts (36).

In the present experiments NAC was added 60 min before the addition of xanthine and xanthine oxidase and was not removed during the period of exposure to free radicals. Therefore, NAC may have prevented the endothelial injury by its direct antioxidant effects. However, we also should consider that it might have increased the intrinsic endothelial mechanisms of defense against oxidative injury.

Our results show that NAC prevents the vascular injury provoked by oxidative stress. The vascular injury is expressed as impairment of endothelium-dependent relaxation and of vascular smooth muscle contraction. Impairment of the NO pathway is more pronounced, with several steps being affected. The EDHF pathway seems to be more resistant to oxidative stress since only the receptor-dependent EDHF-mediated relaxation was affected.

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