Antioxidative Properties of Acetylsalicylic Acid on Vascular Tissues From Normotensive and Spontaneously Hypertensive Rats

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Background—The mechanisms of the beneficial cardiovascular effects of acetylsalicylic acid (ASA, aspirin) therapy are not completely understood. Oxidative stress and inflammation play important roles in the development of cardiovascular diseases.

Methods and Results—In this study, we tested the hypothesis that ASA treatment could reduce superoxide anion (O$_2^-$) generation in aortic ring and in cultured aortic smooth muscle cells (SMCs) from normotensive (WKY) and hypertensive (SHRs) rats by means of the Lucigenin-enhanced chemiluminescence method. Although ASA did not show any short-term effect in vitro and in vivo, long-term oral treatment (100 mg/kg/day, 12 days) significantly reduced the basal O$_2^-$ production by 27% and 45% in aorta of normotensive and hypertensive rats, respectively, in association with a reduction of the NAD(P)H oxidase activity in both groups. These effects were dose-dependent from 10 to 100 mg/kg/day. Similar effects were observed in SMCs following long-term incubation (48 hours) with ASA. ASA treatment also completely inhibited the angiotensin II–induced hypertension and O$_2^-$ production. Moreover, ASA treatment significantly improved the impaired aortic relaxation response to acetylcholine and markedly attenuated the age-dependent development of hypertension in SHRs.

Conclusion—Long-term ASA treatment in vivo markedly reduced vascular O$_2^-$ production through lowering the NAD(P)H oxidase activity in both normotensive and hypertensive rats. These antioxidative properties of ASA are likely involved in the restoration of aortic vasorelaxation, in the attenuation of the development of hypertension in young SHRs, and in the prevention of hypertension following long-term angiotensin II infusion. (Circulation. 2002;105:387-392.)

Key Words: aspirin | superoxide | hypertension | angiotensin II
Methods

Animals
Studies were performed in male Sprague-Dawley (SD, 250 g), Wistar-Kyoto (WKY), and spontaneously hypertensive rats (SHRs) (Charles River Laboratories, St Constant, Quebec, Canada). All experimental procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and monitored by an institutional care committee. The animals were given free access to drinking water. For control rats, drinking water was free of any drug, whereas ASA or ibuprofen was added in the drinking water of treated rats. For Ang II–treated groups, osmotic pumps (model 2000, Alza Corp) containing normal saline (sham) or Ang II were implanted subcutaneously in the SD rats as described before.6 At the end of the treatment, the thoracic aorta was excised and cut into either 2-mm ring segments for O$_2^\cdot$ measurement or 4-mm ring for isometric tension studies.

Protocols

Studies in Sprague-Dawley Rats

Short-Term Effect of ASA on Superoxide Production
Four SD rats without any drug treatment were euthanized and the aortic rings were incubated in vitro with 10$^{-4}$ mol/L of ASA at room temperature for 10 minutes before O$_2^\cdot$ determination.

Time-Effect and Dose-Effect of ASA on Aortic Superoxide Production
ASA treatment (100 mg/kg/day) was performed in 12, 4, 4, 4, and 5 SD rats for 0, 3, 6, 9, and 12 days, respectively. The dose-effect of ASA was evaluated by comparing animals treated either with water (control, n=9) or with one of four doses of ASA (10, 25, 50, and 100 mg/kg/day) for 12 days in 4, 5, 4, and 5 SD rats, respectively.

Effect of Ibuprofen on Aortic Superoxide Production
SD rats were treated either with water (control, n=6) or ibuprofen (100 mg/kg/day, n=6) for 12 days. Aortic O$_2^\cdot$ production was measured with 5 μmol/L Lucigenin.

Effect of ASA on Ang II–Induced Superoxide Production and Hypertension
Sixteen SD rats were separated into sham (n=6), Ang II alone (n=5), and Ang II+ASA-treated (n=5) groups. Ang II was delivered by the subcutaneous osmotic pumps at the rate of 200 ng/kg/min for 12 days. ASA (100 mg/kg/day) was added in drinking water for simultaneous treatment in Ang II+ASA group. The systolic blood pressure (BP) was measured with tail cuff plethysmography (Harvard Apparatus Ltd) before and every other day during the treatment. Aortic O$_2^\cdot$ production was determined with 5 μmol/L Lucigenin.

Studies in WKY and SHRs
The effects of ASA on aortic superoxide production and vasorelaxation to acetylcholine were studied in 12-week-old WKY and SHRs separated into ASA-treated (100 mg/kg/day for 12 days, 7 WKY and 10 SHRs) and control (8 WKY and 10 SHRs) groups. The effect of ASA on the development of hypertension was performed in 6-week-old WKY (n=6) and SHRs (n=6). Both species were separated into ASA (100 mg/kg/day, n=3) and control (n=3) groups and were treated for 53 days. BP was measured regularly by tail cuff plethysmography. At the end of treatment, systolic blood pressure was also measured directly through a femoral artery catheter in anesthetized rats.

Studies in Cultured Aortic Smooth Muscle Cells
Aortic SMCs from 12-week-old WKY (n=3) or SHRs (n=3) were isolated and cultured as described previously.6 The drug solution (final concentration 10$^{-3}$ mol/L, pH=7.4) or equal volume of buffer (control) was added into cell culture medium and cultured with SMCs either for 10 minutes (short-term) or for 48 hours (long-term) at 37°C. The cells were thereafter mechanically scraped and washed twice by centrifugation to remove cell debris and free drug. These cells were immediately used for O$_2^\cdot$ measurement.

Superoxide Anion Measurement
The O$_2^\cdot$ production was measured using the Lucigenin-enhanced chemiluminescence method as described previously.7 Briefly, after 10 minutes equilibration in Krebs-HEPES buffer at room temperature, the aortic ring was transferred to a scintillation vial containing 250 μmol/L Lucigenin for determining the basal O$_2^\cdot$ level. For cultured SMC study, about 10$^5$ cells were added into the counting vials. The chemiluminescence was recorded by a liquid scintillation counter (Wallac 1409). At the end of the count, the fresh aortic ring was weighed and the total SMCs protein was determined. Recently, the chemiluminescence technique using high concentration of Lucigenin has been criticized because reduced Lucigenin was reported to generate O$_2^\cdot$.8 To validate data obtained with high concentration of Lucigenin, low concentration of Lucigenin (5 μmol/L) was used in additional groups.

To evaluate NAD(P)H oxidase activity, high concentration of NADH (100 μmol/L) was added into the counting vial containing 250 μmol/L of Lucigenin and aortic rings or SMCs to evaluate the NADH-activated O$_2^\cdot$ production. This measure was also validated by using an NAD(P)H oxidase inhibitor, diphenylene iodonium (DPI, 100 μmol/L). Aortic rings were incubated with DPI for 10 minutes at room temperature before the basal O$_2^\cdot$ production was evaluated with 5 μmol/L of Lucigenin. The DPI-inhibitable O$_2^\cdot$ production, which represents the intrinsic NAD(P)H oxidase activity, was expressed as the difference of aortic basal O$_2^\cdot$ production in the presence and in the absence of DPI.

Isometric Tension Studies
Aortic rings (4 mm) from 6 control and 6 ASA-treated 12-week-old WKY and SHRs were suspended in individual organ chambers filled with Krebs buffer solution and were submaximally precontracted with 10$^{-6}$ mol/L of phenylephrine. After a stable contraction plateau was reached, the rings were exposed to cumulative acetylcholine concentrations (Ach, 10$^{-9}$ to 10$^{-5}$ mol/L) to determine the endothelium-dependent relaxation. Responses to Ach were expressed as a percent of the precontracted tension of phenylephrine. To prevent the synthesis of prostaglandins and their influence on the vasorelaxant response, all studies were performed in the presence of 10 μmol/L of indomethacin.

Data Analysis
Data are expressed as mean±SEM. Statistical comparisons were made by Student’s t test for paired groups data or one way ANOVA followed by Tukey HSD analysis for multivariance. The vasorelaxation responses and the levels of blood pressure were analyzed by repeated measures ANOVA.

Results

Short-Term Effect of ASA on Aortic Superoxide Production In Vitro
Aortic rings from SD rats (n=4) were incubated with or without 10$^{-4}$ mol/L of ASA at room temperature for 10

![Figure 1. Time course of the effect of ASA treatment (100 mg/kg/day) on basal aortic O$_2^\cdot$ production in SD rats. **P<0.01 vs untreated rats.](image-url)
minutes and \( \cdot \text{O}_2 \) production was evaluated thereafter. The basal \( \cdot \text{O}_2 \) production by untreated rings was 4.5 ± 0.1 cpm/10^3/mg tissue, and the incubation with ASA did not modify this value (4.3 ± 0.2 cpm/10^3/mg tissue).

**Time-Effect and Dose-Effect Relationship of ASA Treatment on Aortic Superoxide Production in Normotensive Rats**

Figure 1 shows that in the first 3 days of treatment, ASA did not produce any significant effect. Subsequently, the \( \cdot \text{O}_2 \) lowering effect of ASA accrued in a time-dependent manner to reach a reduction of 48.5% at the end of the 12-day treatment. Figure 2A shows that ASA treatment produced a dose-dependent decrease in the aortic basal \( \cdot \text{O}_2 \) production. The basal \( \cdot \text{O}_2 \) level in control rats (4.7 ± 0.1 cpm/10^3/mg tissue) was progressively and significantly decreased with 10, 25, 50, and 100 mg/kg/day ASA treatments, respectively (\( P < 0.01 \)). A similar dose-related inhibitory effect of ASA was also observed on the NADH-activated \( \cdot \text{O}_2 \) production (Figure 2B) (\( P < 0.01 \)). The pattern of these dose-effect curves indicated that a maximum effect was reached at about the dose of 100 mg/kg/day, which was chosen for all following studies.

The effects of ASA (100 mg/kg/day) on aortic basal \( \cdot \text{O}_2 \) production and on NAD(P)H oxidase activity were further evaluated with lower concentration of Lucigenin (5 μmol/L) in 10 SD rats. The results illustrated in Figure 3 showed similar effects of ASA treatment on basal \( \cdot \text{O}_2 \) production (decrease of 32%, \( P < 0.001 \), \( n = 5 \)) and on the NAD(P)H oxidase activity (reduction of 34% of the DPI-inhibitable \( \cdot \text{O}_2 \) production, \( P < 0.001 \), \( n = 5 \)) as those estimated with higher concentration of Lucigenin.

To evaluate whether the inhibitory effect of ASA on \( \cdot \text{O}_2 \) production is mediated by the inhibition of the COX enzymes, the effect of ibuprofen, a nonselective COX inhibitor, was also studied. The oral treatment with ibuprofen (100 mg/kg/day) for 12 days did not modify the basal aortic \( \cdot \text{O}_2 \) production (ibuprofen 1.9 ± 0.4 versus control 1.8 ± 0.1 cpm/10^3/mg tissue).

**Effects of ASA Treatment on Aortic Superoxide Production in Hypertensive Rats**

Twelve-week-old SHRs and WKY rats were treated with ASA (100 mg/kg/day) for 12 days. The aortic basal \( \cdot \text{O}_2 \) production was 3.8 ± 0.1 and 7.1 ± 0.3 cpm/10^3/mg tissue and the NADH-activated \( \cdot \text{O}_2 \) production was 136.7 ± 8.8 and 186.6 ± 7.0 cpm/10^3/mg tissue in control WKY and SHRs, respectively (\( P < 0.01 \), SHRs versus WKY). ASA treatment decreased the basal \( \cdot \text{O}_2 \) production by 27% and 45% in WKY and SHRs, respectively (\( P < 0.001 \) versus untreated rats) (Figure 4A). A similar decrease of 25% and 51% in NADH-activated \( \cdot \text{O}_2 \) production was also observed in ASA-treated WKY and SHRs, respectively (\( P < 0.01 \)) (Figure 4B). It is noticeable that ASA treatment completely restored to normal the higher basal and activated \( \cdot \text{O}_2 \) production in SHRs. The ASA treatment did not modify the BP of these WKY or SHRs.

**Inhibitory Effect of ASA on Ang II–Induced High Level of Superoxide Production and Hypertension**

Ang II infusion increased by 77% the aortic basal \( \cdot \text{O}_2 \) production from the control level of 1.6 ± 0.1 to 2.9 ± 0.4 cpm/10^3/mg tissue (\( P < 0.05 \)) and by 44% the BP from the control level of 135 ± 2 to 194 ± 9 mm Hg in SD rats. Simultaneous treatment of Ang II–infused rats with ASA (100 mg/kg/day) completely inhibited \(( P < 0.05 \)) the Ang II–induced high level of \( \cdot \text{O}_2 \) production, which remained at 1.8 ± 0.1 cpm/10^3/mg tissue, as well as the BP rise because the BP remained at 139 ± 4 mm Hg (\( P < 0.01 \)).

**Effects of ASA on Superoxide Production in Cultured Aortic SMCs**

The basal \( \cdot \text{O}_2 \) production that was 36.7 ± 2.7 and 54.7 ± 2.5 cpm/μg protein in untreated cells from WKY and SHRs, respectively (\( P < 0.001 \), SHRs versus WKY), was respectively decreased by 60.2% and 73.1% after long-term incu-

![Figure 2](http://circ.ahajournals.org/)

![Figure 3](http://circ.ahajournals.org/)
bation with ASA for 48 hours \( (P<0.01) \) (Figure 4C). The NADH-activated \( O_2^- \) production, which was \( 41.8 \pm 1.4 \) and \( 89.9 \pm 9.9 \) cpm/10^7 \( \mu \)g protein in control cells from WKY and SHRs \( (P<0.001, \text{SHRs versus WKY}) \), was respectively lowered by \( 54.4\% \) and \( 53.3\% \) in ASA-treated cells \( (P<0.01 \text{ versus untreated}) \) (Figure 4D). The incubation of SMCs with ASA for 10 minutes did not modify the \( O_2^- \) production in those cells (data not shown).

**Effect of ASA on Acetylcholine-Induced Vasorelaxation in Aortic Rings From Normotensive and Hypertensive Rats**

The dilatory dose-response curves of the aorta to Ach indicated that the endothelium-dependent relaxation was impaired in aorta from SHRs (maximal relaxation of \( 14.4 \pm 0.8\% \) compared with \( 26.9 \pm 0.9\% \) in untreated WKY rats; \( P<0.01 \)), whereas the sensitivity to Ach, as reflected by the \( EC_{50} \), was not altered (Figure 5). The long-term ASA treatment partially restored the impaired relaxation in SHRs by increasing the maximal response to \( 20.6 \pm 0.8\% \) \( (P<0.01 \text{ versus untreated SHRs}) \). However, no change in the relaxation was induced by ASA in WKY rats.

**Preventive Effect of ASA Treatment on the Development of Hypertension in Young SHRs**

Six-week-old SHRs and WKY rats were treated with ASA \( (100 \text{ mg/kg/day}) \) or water for 53 days. At the beginning of the study, there was no difference in BP between WKY and SHRs. Thereafter, the BP increased progressively in untreated SHRs from an initial value of \( 141 \pm 3 \) to \( 216 \pm 2 \) mm Hg (75 mm Hg increase) at the end of study (Figure 6). In ASA-treated SHRs (SHR-ASA), the rise of the BP was significantly attenuated with an increase of only \( 45 \) mm Hg (from \( 141 \pm 6 \) to \( 186 \pm 4 \) mm Hg) at the end of 53-day treatment \( (P<0.001 \text{ versus untreated SHRs}) \). In contrast, ASA treatment had no effect on BP in age-matched WKY rats. At the end of treatment, the systolic BP measured by direct femoral artery cannulation was significantly lower in ASA-treated SHRs than that in untreated SHRs \( (183 \pm 4 \text{ versus } 217 \pm 2 \text{ mm Hg}, P<0.001) \). ASA treatment did not

![Figure 4. Effects of ASA treatment on basal (A and C) and NADH-activated (100 \( \mu \)mol/L; B and D) \( O_2^- \) production in aortic rings and cultured aortic smooth muscle cells in WKY and SHRs. **\( P<0.01 \) vs untreated rats and +\( P<0.01 \) vs WKY rats.](image)

![Figure 5. Effects of ASA treatment (100 mg/kg/day, 12 days) on dose-response vasodilatory effects of acetylcholine in aortic rings from WKY and SHRs. Relaxation was expressed as percent of the precontracted tension induced by phenylephrine \( (10^{-7} \text{ mol/L}) \). **\( P<0.01 \).](image)

![Figure 6. Effects of ASA treatment (100 mg/kg/day) on the evolution of blood pressure in WKY and SHRs. The drug treatment was started at the age of 6 weeks (day 0) and lasted for 53 days. **\( P<0.01 \).](image)
modify the body weight gain of SHRs and WKY rats (data not shown).

Discussion

The major findings in this study are as follows: (1) long-term ASA treatment dose dependently (10 to 100 mg/kg/day) reduced aortic $O_2^-$ production presumably through lowering the tissue NAD(P)H oxidase activity in both normotensive and hypertensive rats as well as in their cultured aortic smooth muscle cells, whereas a long-term treatment with ibuprofen did not show any effect; (2) ASA treatment completely inhibited the Ang II–induced superoxide production and BP elevation; (3) ASA treatment improved significantly the impaired Ach-induced aortic relaxation in SHRs, although this treatment had no effect on vasodilatory functions in WKY rats; and (4) long-term ASA treatment initiated in young still normotensive SHRs significantly attenuated the subsequent age-dependent development of hypertension; however, the ASA treatment did not exert a hypotensive effect in age-matched WKY or in 12-week-old SHRs once the hypertension was established.

The present results are the first direct evidence that ASA treatment can decrease aortic tissue $O_2^-$ production through lowering local tissue NAD(P)H oxidase activity. The inhibitory effects of ASA, evaluated with high concentration (250 $\mu$mol/L) of Lucigenin and NADH, were confirmed with lower concentrations of Lucigenin (5 $\mu$mol/L).

The molecular mechanism underlying these inhibitory effects of ASA on the NAD(P)H oxidase remains unknown. Our results indicated that ASA itself could not directly inhibit this oxidase because short-term incubation of aortic rings or cultured SMCs with high concentrations of ASA in vitro did not modify the $O_2^-$ production. In addition, the observations that ASA treatment needed to be pursued for at least 3 days (in vivo) or 2 days (in cultured SMCs) to produce its effects do not support a direct inhibition of the oxidase by ASA.

Inflammatory reactions can constitute a significant source of oxidative stress and damage by markedly raising the number of activated leukocytes that can rise the quantity of reactive oxygen species. ASA is a potent antiinflammatory drug and inhibitor of COX. However, our finding that ibuprofen, another antiinflammatory and nonselective COX inhibitor, did not modify the $O_2^-$ production suggests that the inhibition of the COX enzymes per se does not account for the antioxidative effects of ASA. Moreover, the antiinflammatory properties of ASA could not completely explain the antioxidative effects observed in control normotensive rats because a state of chronic inflammation is not normally expected to occur in this group. The effects of ASA in cultured aortic SMCs also suggest that ASA can exert its antioxidative effects on those cells independent of leukocyte infiltration and inflammation. The similar degree of inhibition of $O_2^-$ production observed in SMCs and in aortic rings by ASA suggests that the antioxidative effect of ASA on SMCs may account for the major effects of ASA on the whole artery and that the antioxidative effects of ASA are independent of blood pressure levels.

Previous studies have demonstrated that long-term Ang II infusion increased the vascular $O_2^-$ production mainly through activation of NAD(P)H oxidase. Our data showed that simultaneous ASA treatment can completely prevent the Ang II–induced $O_2^-$ production, thus supporting the potent antioxidants properties of ASA. The reduction of the NAD(P)H oxidase activity by ASA could represent the major mechanism underlying this effect.

In pathological conditions, excessive production of $O_2^-$ reacts with nitric oxide (NO), and reduces the level of this vasodilator while simultaneously increasing the levels of tissue-damaging peroxynitrite, which can impair further the vascular relaxation mechanisms. The present study showed that ASA treatment significantly restored the impaired vasorelaxation response to Ach only in SHRs characterized by high aortic $O_2^-$ production, but did not modify the vasorelaxation in normotensive WKY rats. These data strongly suggest that ASA treatment improved endothelium-dependent vasorelaxant functions in SHRs mainly by its antioxidant properties, thus increasing the bioavailability of NO in response to Ach. The lack of effect of ASA on Ach-dependent relaxation in healthy WKY rats suggests that under normal conditions the basal superoxide production does not influence the vasorelaxation of aorta.

Another important finding is that ASA administered to young, still-normotensive SHRs significantly attenuated the time-dependent development of hypertension. The hypotensive effect of ASA was not observed in age-matched WKY nor in 12-week-old hypertensive and normotensive rats, although their $O_2^-$ level was equally reduced by ASA. These results indicate that long-term ASA treatment does not exert a direct hypotensive action. In contrast to the present observation, Schirner et al have reported that ASA oral treatment could reduce or increase BP in SHRs depending on their initial BP level being above or below 160 mm Hg, respectively. On the other hand, Taube et al observed that a single intravenous injection of ASA resulted in a short-lasting decrease of BP, but repeated injection caused a slight increase of BP in male SHRs. The possible explanations for these controversies might be due to difference in the age, BP, sex, the route of administration, and the dose of ASA.

Our previous results have shown that there is a positive linear relationship between the arterial $O_2^-$ and the blood pressure level in SHRs and that the hypertension developed in parallel with the rise in $O_2^-$ production in that model. Several other studies have also suggested that enhanced $O_2^-$ production plays an important role in the development of hypertension. Taken together, the present data suggest that ASA attenuated the development of hypertension in young SHRs probably through its antioxidative properties. It is noticeable that, although the higher production of $O_2^-$ in SHRs was completely normalized by the ASA treatment, only partial prevention of hypertension was achieved. This observation indicates that the oxidative stress is not the unique etiological factor in the development of hypertension in that model. However, the fact that ASA completely prevented the development of hypertension in the Ang II–infused rats suggest that the Ang II–induced hypertension model is mainly dependent on enhanced oxidative stress.

In the present study, ASA produced a significant decrease of 19% ($P<0.01$) in aortic $O_2^-$ production at a dose as low as
10 mg/kg/day. Depending on the interspecies dosage conversion factors based on equal body surface (7:1 for conversion from rat to human), the dose of 10 mg/kg for rats can be converted to 1.43 mg/kg for human or 100 mg for a 70 kg human. This dose is within the range of low dose of ASA used for the prevention of cardiovascular diseases.

In conclusion, the present results indicate that long-term ASA treatment importantly reduced vascular O$_2^-$ production by lowering the NAD(P)H oxidase activity in both normotensive and hypertensive rats and in cultured SMCs. ASA treatment also completely inhibited Ang II–induced O$_2^-$ production and hypertension. These effects of ASA cannot be explained only by its inhibition of the COX enzymes and its antiinflammatory properties. The antioxidative properties of ASA are presumably involved in the effect of the drug to restore the impaired endothelium-dependent vasorelaxation and to attenuate the development of hypertension in SHRs. These findings indicate that ASA treatment constitute an effective strategy for antioxidative therapy by reducing tissue O$_2^-$ production and also provide a new insight into the understanding of the beneficial cardiovascular effects of ASA.

**Limitation of Study**

This study described observations in relation to the development of 2 experimental models of hypertension in the rat, which may not mimic human essential hypertension. Similar antioxidative properties of ASA need to be demonstrated in humans before postulating such a mechanism in the cardioprotective effect of long-term ASA therapy in humans.

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