The role of endothelium-derived contracting factor or factors in modulating relaxations and contractions to adenine nucleotides was examined in aortas from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) and Wistar rats. During contractions to phenylephrine, the relaxations to ATP were impaired significantly in SHR compared with WKY aortas with endothelium. In rings treated with N⁶-nitro-l-arginine (to inhibit nitric oxide synthase), the endothelium significantly augmented contractions evoked by ATP; this enhancement was greater in SHR compared with WKY aortas. Indomethacin (inhibitor of cyclooxygenase) and SQ 29,458 (antagonist of thromboxane/prostaglandin endoperoxide receptors) but not dazoxiben (inhibitor of thromboxane synthase) significantly augmented the maximal relaxation in WKY rats, abolished the impairment of the relaxation in SHR, and prevented the potentiation by the endothelium of the contractions evoked by ATP. In older animals (10 to 12 months old), the endothelium-dependent concentration-relaxation curves to ATP in SHR and WKY aortas treated with indomethacin were superimposable, as were the concentration-contraction curves (with N⁶-nitro-l-arginine present). Endothelium-dependent concentration-relaxation and -contraction curves to ADP obtained in these preparations overlapped also. In Wistar rats, the magnitude of the endothelium-dependent relaxations to either ATP or ADP were significantly smaller compared with the other strains, and the endothelium-dependent contractions were even smaller. Results show that adenine nucleotides stimulate the production of both endothelium-derived relaxing and contracting factors. Although there is no obvious age-related alteration in the capacity of aortas to release endothelium-derived relaxing factor, aging enhances endothelium-derived contracting factor activity in WKY rats. (Hypertension. 1993;22:577-583.)

KEY WORDS • endothelium-derived contracting factors • prostaglandin endoperoxides • hypertension • aging • nitric oxide • purines • receptors, purinergic
In parallel experiments, either ATP, ADP, or α,β-methylene ATP was used to inhibit cyclooxygenase and contracted with phenylephrine (3 x 10^{-6} mol/L). In some preparations, N^0-nitro-L-arginine caused contractions that exceeded 10% of the response to 60 mmol/L KCl; the experiments were discontinued, and only aortas in which the nitric oxide synthase inhibitor evoked marginal contractions were used to study the response to ATP. In some experiments, for determination of the involvement of endothelium-derived contracting factor, the rings were incubated for 40 minutes with either indomethacin (10^{-5} mol/L), dazoxiben (10^{-4} mol/L, inhibitor of thromboxane synthase), or SQ 29,458 (10^{-6} mol/L, antagonist of thromboxane/prostaglandin endoperoxide receptors) before the addition of adenine nucleotides.

### Drugs and Chemicals
The following drugs were used: acetylcholine hydrochloride, ADP sodium salt, ATP Tris salt, α,β-methyl-ene ATP lithium salt, indomethacin, phenylephrine hydrochloride (all from Sigma Chemical Co, St Louis, Mo), dazoxiben (Pfizer, Groton, Conn), N^0-nitro-L-arginine (Aldrich Chemical Co, Milwaukee, Wis), and SQ 29,458 ([1S-[1a,2b(5Z),3j8,4a]]-7-[3-[[2-[(phenylamino)carbonyl]hydrizinyl[methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid; ER Squibb & Sons Inc, Princeton, NJ). Drugs were prepared in water except for indomethacin (dissolved by sonication in water and Na_2CO_3, which had no effect at the final bath concentration of 5 x 10^{-4} mol/L) and SQ 29,458 (dissolved in dimethyl sulfoxide; final bath concentration, 0.1% vol/vol).

### Statistical Analysis
In each experimental group, n refers to the number of animals from which aortas were taken. Results are shown as mean±SEM. Relaxations are expressed as percent decreases in tension from the plateau-contraction evoked by phenylephrine (3 x 10^{-4} mol/L). The contractions evoked by the adenine nucleotides under basal conditions are expressed as the percentage of the maximal response to 60 mmol/L KCl obtained at the beginning of the experiment before drug treatment. The potency of agonists was determined as the pD_2, which is the negative logarithm of the concentration causing half the maximal response. The maximal contraction to ATP could not be determined under the experimental conditions used, so its potency was estimated in terms of the EC_50, which is the negative logarithm of the concentration of ATP causing a contraction equal to 20% of that evoked by 60 mmol/L KCl in the same tissue. Statistical comparisons were performed by means of an analysis of variance or Student's t tests for paired and unpaired observations. Differences were considered to be statistically significant at a value of P<0.05.

### Results
#### ATP: Relaxations
Five-month-old WKY rats and SHR. In WKY aortas with endothelium, the cumulative addition of ATP (10^{-3} to 10^{-2} mol/L) during contractions to phenylephrine (3 x 10^{-4} mol/L) caused concentration-dependent relaxations that were maximal at 10^{-4} mol/L (Fig 1). In SHR...
aortas, ATP also evoked concentration-dependent relaxations; however, higher concentrations of the agonist caused a rebound contraction (Fig 1). In both WKY and SHR aortas, the relaxations evoked by ATP were abolished by the removal of the endothelium and in the presence of N\textsuperscript{G}-nitro-L-arginine (10\textsuperscript{-4} mol/L, data not shown). In WKY rings with endothelium incubated with either indomethacin (10\textsuperscript{-5} mol/L), SQ 29,458 (10\textsuperscript{-6} mol/L), or dazoxiben (10\textsuperscript{-4} mol/L), the contractions to phenylephrine were reduced significantly (see Table 2). In these preparations, the maximal relaxation to ATP was increased significantly by both indomethacin or SQ 29,458 but not by dazoxiben (Fig 1). In SHR aortas with endothelium, the inhibitors of the arachidonic acid cascade did not affect significantly the contractions evoked by phenylephrine (Table 2); both indomethacin and SQ 29,458 inhibited the rebound in tension induced by higher concentrations of ATP, which then caused further relaxation (Fig 1). The concentration-relaxation curves to ATP obtained in the presence of dazoxiben were not significantly different from control (Fig 1).

**Twelve-month-old SHR and WKY and Wistar rats.** In aortas of 12-month-old rats, the contractions evoked by phenylephrine (3×10\textsuperscript{-6} mol/L) did not differ significantly among the three strains. The concentration-relaxation curves to ATP obtained under these conditions were superimposable for the lower concentrations of the agonist (Fig 2). However, the maximal response to ATP was significantly greater in preparations from SHR and WKY rats than in those from Wistar rats (Fig 2). The maximal responses and pD\textsubscript{2} values obtained in mature SHR and WKY rats did not differ significantly from those obtained in younger animals (Table 3 and Figs 1 and 2).

**ATP: Contractions**

Five-month-old WKY rats and SHR. The contractions induced by 60 mmol/L KCl did not differ significantly among groups even after endothelium removal (see Table 2). In the presence of N\textsuperscript{G}-nitro-L-arginine (10\textsuperscript{-4} mol/L), the cumulative addition of ATP elicited concentration-dependent contractions in rings with endothelium from both WKY rats and SHR (Fig 3). The EC\textsubscript{50} of ATP was greater in aortas from SHR than in those from WKY rats (Table 3). Removal of the endothelium induced a shift to the right of the concentration-contraction curves to ATP in preparations from both strains; the interspecies difference between WKY rats and SHR was no longer significant. In WKY rings with endothelium treated with either indomethacin (10\textsuperscript{-5} mol/L) or SQ 29,458 (10\textsuperscript{-6} mol/L), there was a significant shift to the right of the concentration-contraction curves to ATP, that in the presence of these inhibitors did not differ significantly from those curves obtained in rings without endothelium (Fig 3). In SHR, indomethacin and SQ 29,458 shifted the concentration-contraction curves to ATP below those obtained in rings without endothelium (Fig 3). In both strains, dazoxiben did not affect the contractions to ATP (data not shown, n=2).

**Twelve-month-old SHR and WKY and Wistar rats.** ATP was equipotent in aortas from SHR and WKY rats and significantly less potent in those from Wistar rats (Table 3) in causing contractions of preparations with

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**TABLE 2. Contractions to Phenylephrine and KCl in Rat Aortic Preparations**

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>Control</th>
<th>Indomethacin, 10\textsuperscript{-6} mol/L</th>
<th>SQ 29,458, 10\textsuperscript{-6} mol/L</th>
<th>Dazoxiben, 10\textsuperscript{-4} mol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>without endothelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=5)</td>
<td>5.4±0.4</td>
<td>4.0±0.5*</td>
<td>4.5±0.3*</td>
<td>4.3±0.4*</td>
</tr>
<tr>
<td>SHR (n=5)</td>
<td>3.9±0.3</td>
<td>3.4±0.2</td>
<td>3.6±0.4</td>
<td>3.6±0.4</td>
</tr>
</tbody>
</table>

**Endothelium**

<table>
<thead>
<tr>
<th></th>
<th>With</th>
<th>With</th>
<th>With</th>
<th>Without</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction to 60 mmol/L KCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY (n=5)</td>
<td>4.4±0.4</td>
<td>4.4±0.5</td>
<td>4.0±0.3</td>
<td>3.5±0.3</td>
</tr>
<tr>
<td>SHR (n=5)</td>
<td>4.4±0.3</td>
<td>4.4±0.7</td>
<td>4.4±0.3</td>
<td>3.7±0.5</td>
</tr>
</tbody>
</table>

WKY indicates Wistar-Kyoto rats; and SHR, spontaneously hypertensive rats. Contractions to phenylephrine were obtained in rings with endothelium only. Results are expressed as mean±SEM.

*Statistically significant differences with responses observed under control conditions.
FIG 2. Plot shows concentration-relaxation curves to ATP in aortas with endothelium from mature Wistar rats (n=4, ■), Wistar-Kyoto (WKY) normotensive rats (n=6, ▲), and spontaneously hypertensive rats (SHR) (n=5, ○). Preparations were incubated with indomethacin (10⁻⁵ mol/L) and contracted with phenylephrine (3×10⁻⁶ mol/L). Relaxations are shown as mean±SEM expressed as percent changes in tension from plateau contraction induced by phenylephrine (4.6±0.5 g in Wistar, 4.3±0.5 g in WKY, 4.0±0.3 g in SHR), which did not differ significantly among strains. †Statistically significant strain-related differences. Removal of the endothelium resulted in a significant shift to the right of the concentration-contraction curves obtained in the three strains (Fig 4). The concentration-contraction curves obtained in the Wistar aortas without endothelium remained to the right of those obtained in WKY and SHR aortas and did not differ significantly (Fig 4).

**ADP**

Relaxations evoked by ADP were investigated in aortas incubated with indomethacin and contracted with phenylephrine (3×10⁻⁶ mol/L) from 12-month-old rats. ADP evoked concentration-dependent relaxations in preparations from SHR and WKY and Wistar rats (Fig 5). The maximal relaxation induced by ADP was comparable in aortas from SHR (53.9±6.9%, n=5) and WKY rats (55.9±12.4%, n=6) but was significantly smaller in Wistar rats (51.5±8.21%, n=4). The pD₂ values (SHR, 6.6±0.1; WKY, 6.1±0.2; Wistar, 5.8±0.2) did not differ significantly among strains. The maximal relaxation to ATP was significantly greater than that induced by ADP in all strains.

In preparations with endothelium treated with N⁶-nitro-l-arginine, ADP evoked concentration-dependent contractions (Fig 5), the maximum of which was significantly greater in aortas from SHR (43.6±6.5%, n=5) and WKY rats (45.1±12.1%, n=6) than in preparations from Wistar rats (12.5±7.2%, n=4). Endothelium removal significantly inhibited the contractions in aortas from the three strains (Fig 5).

**α,β-Methylene ATP**

In aortas with endothelium (incubated with 10⁻⁵ mol/L indomethacin and contracted with 3×10⁻⁶ mol/L phenylephrine) taken from 12-month-old SHR, the cumulative addition of α,β-methylene ATP (10⁻⁸ to 10⁻⁴ mol/L; a stable analogue of ATP) did not cause relaxation but induced further increases in tension during contraction to phenylephrine (Fig 6).

In preparations incubated with 10⁻⁴ mol/L N⁶-nitro-l-arginine, α,β-methylene ATP induced concentration-dependent contractions that were larger in rings with than in those without endothelium (Fig 6). In the presence of indomethacin, the contractions to α,β-methylene ATP were significantly reduced in rings with endothelium; at 3×10⁻³ mol/L, they averaged 20.0±2.7% of the response to 60 mmol/L KCl in the presence of indomethacin compared with 39.0±6.7% in control (n=3). The contractions obtained in the presence of the inhibitor of cyclooxygenase did not differ significantly from those obtained in rings without endothelium.

**Discussion**

The results indicate that the endothelium-dependent relaxations to ATP are impaired in aortas from SHR by the production of an endothelium-derived contracting factor. To further characterize this effect of ATP, experiments determined (1) whether the production of endothelium-derived contracting factor induced by ATP in the aorta of the WKY rat augments with age as shown previously for acetylcholine; other experiments also (2) assessed whether or not this augmentation is accompanied by an increase in the sensitivity of endothelial cells to ATP, (3) assessed the relaxations and contractions to ADP, and (4) compared the responses to ATP in SHR and WKY rats with those in Wistar rats of the same age to further document strain-related differences. Finally, the question was addressed whether or not the endothelium modulates the contraction of vascular smooth muscle induced by α,β-methylene ATP, which is known to be endothelium independent in most blood vessels, including the rat aorta.

The impairment of the relaxation to ATP in rat aorta was not reported in previous studies, possibly because these authors only considered lower concentrations of ATP, for which there is no significant rebound in tension.
as shown in the present study. However, a previous study in the rat showed an impairment of the endothelium-dependent relaxations to ATP in carotid arteries from SHR and aged WKY rats, although the mechanism involved in the reductions was not determined. The present study demonstrates that the impairment is prevented in the presence of inhibitors of cyclooxygenase or of thromboxane A<sub>2</sub>/endoperoxide receptors but not of thromboxane synthase. Therefore, the endothelium-derived contracting factor involved in this impairment is very likely to be an endoperoxide, as is the case for the reduction of endothelium-dependent relaxations to acetylcholine. Endothelium-derived contracting factor mediates the potentiation by the endothelium of the contractions evoked by ATP in aortas treated with the nitric oxide synthase inhibitor N<sup>o</sup>-nitro-L-arginine. Indeed, like the endothelium-dependent contractions to acetylcholine, the latter component of the contraction elicited by ATP is prevented in the presence of inhibitors of cyclooxygenase or of thromboxane A<sub>2</sub>/endoperoxide receptors but not of thromboxane synthase.

To judge from the increase in endothelium-dependent contractions, there is an enhanced production and/or action of endothelium-derived contracting factor with age. It has been suggested that this may be correlated with blood pressure elevation. Although there was indeed a significant increase of blood pressure in WKY rats, it still remained below the levels obtained in 5-month-old SHR. Because the endothelium-dependent contractions in old WKY rats are greater than those obtained in 5-month-old SHR, the moderate elevation of blood pressure in WKY rats may not account fully for the obtained potentiation.

The endothelium-dependent relaxations obtained in the presence of indomethacin were comparable in both strains and did not differ significantly from those observed in the younger animals under identical conditions. Therefore, the increase of endothelium-dependent contractions with age in WKY rats and hypertension in SHR is not a consequence of a nonspecific increase in the sensitivity of endothelial cells to ATP. Rather, because the induction of the production of endothelium-derived relaxing factor and prostanoids by adenine nucleotides is dependent on the mobilization of intracellular calcium, the lack of significant age-related alteration of the endothelium-dependent relaxations suggests that this increase may reflect an enhanced metabolism of arachidonic acid.

The endothelium-dependent responses, contractions and relaxations, to ADP obtained in aortas of old WKY rats and SHR, respectively, were comparable. However, in terms of maximal response (relaxation or contraction), there was a significant strain-related difference between curves.
tion), ADP was less efficacious than ATP in both strains. This difference has been documented previously for endothelium-dependent relaxations caused by adenine nucleotides and may be attributed to nucleotide receptor heterogeneity in the rat aorta. Thus, P₂-purinoceptors present in endothelial cells mediate the relaxation to ADP, whereas in addition, relaxations to ATP involve the so-called nucleotide receptors, which are activated poorly by ADP. The present results show that ADP evokes the production of endothelium-derived contracting factor, which then explains the impairment of the relaxations to this purine in the SHR aorta reported previously. 

In aortas from aged Wistar rats, the endothelium-dependent contractions obtained with both ATP and ADP were significantly less than in preparations from aged SHR and WKY rats. Similarly, the direct stimulation of vascular smooth muscle in rings without endothelium and the endothelium-dependent relaxations were also smaller in comparison with the same effects in aortas from SHR and WKY rats. Whether this reduced responsiveness results from in vivo desensitization, by virtue of a greater endogenous stimulation, or stems from genetic differences in receptor density and/or coupling cannot be inferred from the data collected in this study.

To judge from the results obtained in aortas from SHR treated with indomethacin, α,β-methylene ATP does not evoke endothelium-dependent relaxation as reported previously in aortas from normotensive rats. These findings suggest that P₂-purinoceptors, if present in endothelial cells, may not be coupled to the mechanisms that elevate cytosolic calcium and trigger the production of both endothelium-derived relaxing factor and prostaglandins, contrary to P₂-purinoceptors and the so-called nucleotide receptors. Nevertheless, the presence of the endothelium potentiated slightly but significantly the contractions to α,β-methylene ATP, an effect mediated by an endothelium-derived contracting factor because it was abolished by indomethacin. Taking into consideration the lack of evidence as yet for endothelial P₂-purinoceptors, this potentiation may reflect a subthreshold, spontaneous release of an endothelium-derived contracting factor.

FIG 5. Plots show concentration-relaxation (left) and concentration-contraction (right) curves to ADP in aortas with (closed symbols) and without (open symbols, right) endothelium from mature Wistar rats (n=4, ▲), Wistar-Kyoto (WKY) normotensive rats (n=6, ■), and spontaneously hypertensive rats (SHR) (n=5, ●). For relaxations, preparations were incubated with indomethacin (10⁻⁵ mol/L) and contracted with phenylephrine (Phe) (3×10⁻⁶ mol/L). Relaxations to ADP (shown as mean±SEM) are expressed as percent changes in tension from plateau contraction to phenylephrine, which did not differ significantly among strains (data not shown). For contractions, after a reference contraction to 60 mmol/L KCl, preparations were incubated with N⁵-nitro-L-arginine (10⁻⁴ mol/L). Contractions to ADP (shown as mean±SEM) are expressed as percent of initial contraction induced by 60 mmol/L KCl in rings with and without endothelium. Reference contraction to 60 mmol/L KCl did not differ significantly among or within strains between rings with and without endothelium (data not shown). *Statistically significant strain-related difference between curves.

FIG 6. Plots show concentration-dependent effects of α,β-methylene ATP in contracted (left) and quiescent (right) aortas with (●) and without (○) endothelium from spontaneously hypertensive rats (n=4). For experiments depicted in the left panel, preparations were incubated with indomethacin (10⁻⁵ mol/L) and contracted with phenylephrine (Phe) (3×10⁻⁶ mol/L). Effects of α,β-methylene ATP (shown as mean±SEM) are expressed as percent changes in tension from plateau contraction to phenylephrine. After reference contraction to 60 mmol/L KCl, preparations were incubated with N⁵-nitro-L-arginine (10⁻⁴ mol/L). Contractions to α,β-methylene ATP (shown as mean±SEM) are expressed as percent of initial contraction induced by 60 mmol/L KCl (5.2±0.5 g in control group, 4.6±0.5 g in rings without endothelium).
In conclusion, the present study demonstrates that ATP and ADP evoke the production of endothelium-derived contracting factor and endothelium-derived relaxing factor in SHR and old WKY aortas. The adenine nucleotides, when released from platelets, endothelial cells, or both, could participate in the regulation of the production of endothelium-derived contracting factor in vivo. The enhanced production of endothelium-derived contracting factor in SHR and old WKY rats may counteract the modulatory function of endothelium-derived relaxing factor and impair the vasodilator action of adenine nucleotides.

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