Age-related changes in carotid vascular responses to adenosine and nitric oxide in the rat: in vitro and in vivo studies

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Omar NM, Marshall JM. Age-related changes in carotid vascular responses to adenosine and nitric oxide in the rat: in vitro and in vivo studies. J Appl Physiol 109: 305–313, 2010. First published May 20, 2010; doi:10.1152/japplphysiol.01245.2009.—We investigated how the ability of adenosine to release nitric oxide (NO) from carotid artery in vitro, and dilator responses evoked in carotid circulation in vivo by systemic infusion of adenosine, change with age in rats of 4–5, 10–12, and 42–44 wk (juvenile, mature, and middle aged). A secondary aim was to follow age-related changes in carotid/cerebral autoregulation. In opened carotid artery, graded doses of adenosine evoked graded increases in NO output measured with a NO sensor that were greater in mature and middle-aged than juvenile rats. Thereafter, the adenosine-evoked increase in cerebral vasomotor conductance (CVC) was unchanged in juvenile and middle-aged rats, yet CBF remained constant in juvenile but increased in middle-aged rats. The NO synthase inhibitor nitro-L-arginine methyl ester (L-NAME; 10 mg/kg iv) increased baseline ABP in all groups but caused larger percentage reductions in baseline CVC and CBF in mature and middle-aged than juvenile rats. Thereafter, the adenosine-evoked increase in CVC was unchanged in juvenile and middle-aged rats, yet CBF remained constant in juvenile but increased in mature, and middle-aged rats. In mature rats, the evoked increases in CVC and CBF were attenuated and further attenuated by L-NAME at 30 mg/kg. We propose that the ability of adenosine to release NO and cause vasodilation in the carotid artery and its circulation is greater in mature, than juvenile or middle-aged rats, but NO has greater tonic dilator influence in carotid circulation of mature and middle-aged than juvenile rats. By middle age, the lower limit of cerebral autoregulation has increased such that the tonic dilator influence of NO on CBF and CVC limits autoregulation of CBF to depressor responses. However, partial NO synthase inhibition overcomes this impairment, raising baseline ABP and allowing adenosine-evoked increases in CVC to increase CBF.

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Adenosine; nitric oxide; vasodilation; cerebral vasculature; ageing

It is accepted that adenosine plays an important role in the regulation of cerebral vasculature. Adenosine concentrations increased two- to sevenfold in rat brain within 30 s of inducing moderate or severe systemic hypoxia with an associated three- to fourfold increase in cerebral blood flow (40). The hypoxia-induced dilatation of pial arteries in the rat was reduced by theophylline, a nonselective adenosine receptor antagonist (23), and by adenosine deaminase (32). Further, the increase in cerebral cortex perfusion induced in the rat by systemic hypoxia was inhibited by topical application of a selective adenosine receptor antagonist that crosses the blood-brain barrier, but not by one that does not cross the blood-brain barrier. Thus the cerebral vasodilator action of adenosine in hypoxia is endothelium dependent (3). It has also been shown that adenosine acting via A2A receptors plays a role in the cerebral dilatation evoked by systemic hypoxia and by systemic hypotension (3, 16, 31).

Consistent with these findings, infusion of adenosine into cerebral circulation induced cerebral vasodilation in the baboon and rabbit (9, 13). Further, dilation induced by adenosine in a range of arteries in vitro and in systemic vascular beds in vivo is at least partly dependent on endothelial synthesis of nitric oxide (NO); it is attenuated by endothelium removal, or inhibition of NO synthesis (see 15, 33). Moreover, tonic and agonist-stimulated synthesis of NO has been implicated in the regulation of cerebral blood flow. Thus NO synthase (NOS) inhibition reduced basal cerebral blood flow and vascular conductance in the rat (2, 28, 35), and reduced the increase in cortical perfusion caused by hypoxia (30) and the cerebral autoregulatory vasodilation induced by systemic hypotension (18, 35). Use of an NO-sensitive electrode provided direct evidence that adenosine releases NO from porcine carotid artery endothelial cells in culture (21) and from rat thoracic and iliac artery (29). However, whether or not adenosine can release NO from arteries that supply cerebral circulation has not been directly tested. There has also been no study of the extent to which NO is involved in cerebral vasodilatation evoked by adenosine in vivo.

The studies mentioned above were all performed on adult animals, no particular attention being paid to age. There are indications that dilator responsiveness to adenosine changes with age. For example, dilator sensitivity to adenosine was lower in aortas from 8 and 12 wk of age than in those of 4 wk (24), whereas the sensitivity fell progressively in aortas from rats of 4–8 wk, 12–16 wk, and 48–72 wk (15), the last group representing extreme old age (see 6). In the coronary circulation, the sensitivity to adenosine was similar at 4–8, 12–16, and 48–72 wk of age, but the magnitude of the dilator responses was smaller at 12–16 and 48–72 wk than at 4–8 wk. Moreover, in both the aorta and coronary circulation, the NO-dependent and NO-independent component of the response to adenosine decreased with age (15).

There have been no comparable studies on the role of NO in adenosine-induced dilation in cerebral arteries, but basilar arteries of the rabbit showed greater sensitivity to adenosine at 24 wk than at 2 or 4 wk of age (35) and the internal carotid artery of the piglet showed much greater adenosine sensitivity at 24 wk than at 1–3 days of age (20). Further, judging from the effects of a selective receptor antagonist, adenosine makes a weak contribution to the cerebral vasodilator response evoked by systemic hypoxia in rats of 6 wk relative to rats of 10–12
wk of age (36, 37). Moreover, cerebral arterioles in rats of 6–8 mo of age showed much greater endothelium-dependent dilator responses to acetylcholine and bradykinin than in those of 22–24 mo, and the disparity was not attributable to age-dependent changes in the sensitivity to NO or in the modulatory influences of vasodilator or vasoconstrictor cyclooxygenase products (22). These results indicated that at least in old age, agonist-induced release of NO is attenuated in cerebral arterial vessels. Clearly, it is impossible to deduce whether disparities between results of such studies reflect differences between species, arteries, and/or the precise age of the animals relative to maturation and life span.

Thus the aim of the present study was to take a first step toward investigating these issues by comparing responses evoked by adenosine in the vasculature that is supplied by the carotid artery in three groups of male Wistar rats at 4–5, 10–12, and 42–44 wk of age (juvenile, mature, and middle aged, respectively). At 4–5 wk, Wistar rats have weaned but are sexually immature. At 10–12 wk they are sexually mature and their body weight is 250–350 g (1), encompassing the weight range used in most published studies on the rat. The life span of the Wistar rat is 2.5–3 yr, which has been compared with human age of ~70–80 yr of age; thus 42–44 wk can be considered to represent a human age of ~40–45 yr (8). We measured the NO released by graded concentrations of adenosine from the endothelial surface of the common carotid artery in vitro by using an NO sensor (see 29). In anesthetized rats, we compared responses evoked by systemic infusion of adenosine on carotid blood flow and vascular conductance before and after NOS inhibition. Adenosine was infused at a dose that reduced mean arterial blood pressure (ABP) to ~60 mmHg, the lower limit of the cerebral autoregulatory range in adult rats (27). Thus these experiments also gave us the opportunity to compare in juvenile, mature, and middle-aged rats the effectiveness of autoregulation in the circulation supplied by the carotid artery, i.e., the ability to maintain blood flow in the face of a fall in ABP. We hypothesized that responses evoked by adenosine in carotid artery in vitro and in carotid circulation in vivo are greater in mature than juvenile or middle-aged rats and that the differences are at least partly explained by parallel changes in the contribution of NO to these responses.

METHODS

All experiments were performed on male Wistar rats supplied by Charles River (Hythe, Kent, UK). They were kept in the Biomedical Services Unit, University of Birmingham, for at least 1 wk before the terminal experiment and were given standard rat chow and water ad libitum. All experiments were approved under the UK Animals (Scientific Procedures) Act 1986 and by the University Biomedical Ethics Review Committee. Three age groups were studied: juvenile (4–5 wk), mature (10–12 wk), and middle aged (42–44 wk).

For all studies, anesthesia was induced with halothane (2.5–3.5%) delivered in O2 at a rate of 3–3.5 l/min into a small box that contained the rat. When the animal was anesthetized as judged by absence of a pedal withdrawal reflex, it was transferred to an operating table.

In Vitro Studies

For these experiments, the animal was killed by overdose of halothane anesthesia delivered via a nose cone, followed by cervical dislocation. The common carotid artery was exposed and carefully dissected from the surrounding tissue. A length of ~2 cm was transected at both ends and placed in Krebs solution at room temperature. Residual connective tissue was cleared with the aid of an operating microscope, and the artery was opened longitudinally by using fine-curved ophthalmic scissors, the blades being directed upward to avoid damage to the endothelial surface.

The carotid artery was then prepared for measurement of NO release, essentially as described by Ray et al. (29). Briefly, the artery was pinned endothelial surface uppermost, onto dental impression material that partially filled a Petri dish of 50-mm diameter. Residual blood was carefully washed away and 10 ml of fresh Krebs was added to the bath. A magnetic flea was placed near the edge of the dish, and the dish was placed on a magnetic stirrer so as to facilitate mixing of solutions added to the bath. The NO sensor (ISO-NOP, World Precision Instruments) with a 2-mm-diameter tip, was held vertically in a micromanipulator and carefully lowered until the tip was in close opposition to the endothelium in the center of the artery, but not actually touching it. The sensor was connected to an NO meter (ISO-NOP Mark II, World Precision Instruments). This set-up was allowed to equilibrate for at least 30 min before the protocol was started. The redox current produced at the electrode was passed across a resistor and continuously recorded as a voltage change with a data-acquisition system (MacLab/2c, AD Instruments; sample rate 100 Hz) connected to a computer (Power Macintosh 6100/60).

Protocol. Experiments were performed on freshly excised carotid arteries of juvenile (130.0 ± 147 g body wt, n = 6), mature (376.2 ± 16.3 g, n = 7), and middle-aged (717.4 ± 32.7 g, n = 7) rats. The NO output was measured in response to graded concentrations of adenosine (100 μm–5 mM) by adding appropriate volumes of a stock solution of adenosine (10 mM). At least 7 min was allowed between additions so that the response to adenosine was completed and the electrode output stabilized again.

For each age group, six to seven control experiments were done by adding different volumes of Krebs, the vehicle for adenosine, to the bath in the same way as adenosine, so as to ensure that the response obtained was the result of addition of adenosine and not the consequence of shear stress (7). Additions of vehicle had no measurable effect on the recorded signal. In four experiments on carotid arteries taken from mature rats, responses evoked by 5 mM adenosine were tested before and 30 min after incubation with the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) at 100 μM. This concentration of L-NAME abolished the adenosine-evoked increase in cGMP in human umbilical vein endothelial cells (34).

On each experimental day, the NO electrode was calibrated by chemical generation of NO (see 29). The regression equation plotted from the calibration curve drawn at the end of the experimental day was used to convert the voltage changes into changes in NO concentration (in nM) for that experimental day only. The sensitivity of the electrode varied from day to day as reported before (see 29). However, as noted in those studies, the range of sensitivity was not great; for example, for the life time of one single electrode membrane that was used for the mature group and half of the juvenile group, it ranged from 0.21 to 0.27 nM/nM (or 2.1–2.7 pA/nM). The electrode calibrations showed comparable linearity for different experimental groups: r = 0.998 ± 0.001, 0.996 ± 0.001, and 0.995 ± 0.003 for juvenile, mature, and middle-aged rats, respectively.

In Vivo Studies

The animals were prepared essentially as we have described previously (29, 36). The inhalation anesthesia was maintained by delivering 2–2.5% halothane in O2 at a rate of 2.5–3 l/min via a nose cone, to allow the right jugular vein to be cannulated for continuous infusion of the steroid anesthetic Saffan (Schering-Plough, 7–12 mg·kg−1·h−1). When the cannula was in place, the inhalation anesthesia was discontinued. Thereafter, a cannula was placed in the trachea so that mucous could be aspirated if required. A cannula was placed in the left femoral artery to allow arterial...
blood pressure to be continuously recorded and samples (150 μl) to be taken at regular intervals for blood gas analysis by using a blood gas analyzer (IL 1640; Instrumentation Laboratories, Warrington, UK). A cannula was placed in the right femoral vein to allow infusion of adenosine and administration of L-NAME (see below). Finally, the left common carotid artery was exposed and cleared so that carotid blood flow (CBF) could be recorded by using a perivascular transonic probe (0.7 V, Transonic Systems) connected to a flowmeter (T106, Transonic Systems).

All cardiovascular variables were recorded on a PowerMac G4 computer via a MacLab/8 (AD Instruments, Hastings, UK) data-acquisition system and chart (AD Instruments) sampling at 100 Hz. Mean arterial pressure and heart rate (HR) were derived from the arterial pressure signal, and carotid vascular conductance (CVC) was computed online as CBF/ABP.

Protocols. Experiments were performed on five different groups of animals. Animals in whom recorded variables were unstable or who showed any respiratory distress were excluded from the study. Arterial blood gases were within normal limits in juvenile, mature, and middle-aged rats (arterial partial pressure of O2 and CO2: 82–89 mmHg and 37–41 mmHg, respectively; pH: 7.39–7.42). Thus the full protocol described below was performed on juvenile (4–5 wk old, 1,251.0 ± 2.9 g, n = 7), mature (10–12 wk old, 321.0 ± 4.5 g, n = 7), and middle-aged rats (42–44 wk old, 506.9 ± 15.9 g, n = 8). Experiments were also performed on two further groups of mature rats (350.0 ± 9.9 g, n = 6; and 330.0 ± 3.6 g, n = 7) that received adenosine infusions before and after higher doses of L-NAME than used in the full protocol (see below).

For all experiments, an equilibration period of at least 30 min was allowed so that the level of anesthesia and all recorded variables could stabilize. Then adenosine was infused by a pump (model 100, Kd Scientifics) for 3 min, at a dose selected to reduce ABP to ~60 mmHg, the lower end of the autoregulatory range (27). The dose used was calculated for individual animals as a dose per kilogram body weight on the basis of pilot experiments: in practice, the dose was adjusted if necessary at the outset of the experiment, so as to achieve the desired effect on ABP. Once the dose had been established, it was used throughout the protocol. Following the infusion, a period of at least 10 min was allowed for all cardiovascular variables to return to steady baselines. A further infusion of adenosine was then made if required. After a further stabilization period of at least 10 min, L-NAME was given at 10 mg/kg iv, and then the whole protocol was repeated.

The dose of 10 mg/kg L-NAME was chosen with the aim of producing maximum inhibition of NOS on the basis that in previous studies, higher doses of L-NAME did not produce greater increases in the baseline level of ABP or in the decreases in baseline femoral vascular conductance, or in the increase in femoral vascular conductance evoked in adenosine or systemic hypoxia (10, 33). Nevertheless, to test whether we had achieved complete inhibition of NOS in carotid circulation, in two further groups of mature rats (see above), adenosine was infused for 3 min as described above, before and after L-NAME at the higher dose of 20 or 30 mg/kg. At the end of the experiments, all animals were killed by overdose of Saffan.

Materials

Adenosine, L-NAME, and the constituents of the Krebs solution were obtained from Sigma-Aldrich (Dorset, UK). Adenosine and L-NAME were dissolved in physiological saline (0.9%). The Krebs solution had the following composition (in mM): 118 NaCl, 4.7 KCl, 25 NaHCO3, 1–2 KH2PO4, 1.1 MgSO4, 1.5 CaCl2, 5.6 glucose, 10 HEPES, and 100 μM l-arginine (29).

Statistical Analysis

All results are expressed as means ± SE. For the in vitro experiments, the response to each dose of adenosine was measured as the difference between the baseline voltage recorded before the adenosine addition and the peak attained within 1 min of agonist addition. This allowed generation of a noncumulative dose-response curve (see 29 and Fig. 1). Repeated-measures ANOVA (group × dose) was used to test the dose-dependent increase in NO release within each group, and factorial ANOVA was used to compare groups; post hoc Scheffé tests were used when appropriate to test the location of effect. For the in vivo experiments, the mean value of each variable during the baseline period before infusion of adenosine was compared with the mean value recorded during the 3-min period of infusion by Student’s paired t-test, both before and after L-NAME. The baseline values of each variable before L-NAME and 15 min after L-NAME administration were also compared by Student’s paired t-test. Comparisons between different groups were made by using ANOVA with Scheffé’s post hoc test when ANOVA indicated significance. In all cases, P < 0.05 was considered statistically significant.

RESULTS

In Vitro Studies

As described by Ray et al. (29) for the aorta, application of adenosine (1 mM) to the isolated carotid artery of juvenile, mature, and middle-aged rats evoked a release of NO that reached a peak within 10–20 s. In each age group, adenosine (100 μm–5 mM) evoked a dose-dependent increase in NO output (Fig. 1). The highest output was achieved at 5 mM, but the dose-response curve did not reach an obvious maximum at 5 mM in any age group. For each age group, the change in NO output over the range of adenosine concentrations was significant, and within groups, the NO output achieved significance at the higher doses of adenosine. The NO output recorded from the carotid arteries of mature rats was significantly greater than that evoked from both the juvenile and the middle-aged rats, the difference between the mature and juvenile groups reaching significance at each of the higher doses of...
adenosine (see Fig. 1). In four arteries taken from mature rats, L-NAME at 100 μM reduced the increase in NO output evoked by 5 mM adenosine from 20 to 0.1 nM (P < 0.05).

In Vivo Studies

There were no significant differences between the baseline values of ABP, CBF, and CVC in the three groups of rats. However, baseline HR was significantly higher in the juvenile than in the mature and middle-aged rats (Table 1).

As indicated in METHODS, adenosine was infused in each animal at a dose that reduced ABP to 60 mmHg. The dose required to achieve this effect was 2.6 ± 0.1 and 2.5 ± 0.1 mg/kg in the juvenile and mature rats, respectively, but only 0.47 ± 0.1 mg/kg in the middle-aged rats, this dose being significantly lower than those used in the other two groups (P < 0.001). In all three groups, ABP fell during adenosine infusion, reaching a plateau level of 60 mmHg within 1 min, and there was an accompanying fall in HR. Concomitantly, there was a significant increase in CVC in all three groups, indicating dilatation in the carotid vascular bed. However, the increase in CVC was greater in the mature rats than in the juvenile and middle-aged rats (Fig. 2). As a consequence, CBF increased during adenosine infusion in the mature rats but not in the other two groups. In fact, CBF was well maintained at the baseline level in all the juvenile rats (see Fig. 2). Mean CBF was also maintained in the middle-aged group, but considering individual middle-aged rats, four of eight showed a decrease in CBF during adenosine infusion; no animals in the other two groups showed a decrease in CBF.

Table 1. Baseline values of cardiovascular variables recorded before and ~15 min after l-NAME (10 mg/kg iv) in juvenile, mature, and middle-aged rats

<table>
<thead>
<tr>
<th></th>
<th>Juvenile</th>
<th>Mature</th>
<th>Middle-Aged</th>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After l-NAME</td>
<td>Before</td>
</tr>
<tr>
<td>ABP, mmHg</td>
<td>115.0 ± 3.3</td>
<td>133.7 ± 2.6†</td>
<td>114.3 ± 3.6</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>537.0 ± 9.5§</td>
<td>490.0 ± 7.7†</td>
<td>403.0 ± 9.9</td>
</tr>
<tr>
<td>CBF, ml/min</td>
<td>1.60 ± 0.18</td>
<td>1.25 ± 0.14*</td>
<td>1.78 ± 0.26</td>
</tr>
<tr>
<td>CVC, ml·min⁻¹·mmHg⁻¹</td>
<td>0.014 ± 0.002</td>
<td>0.009 ± 0.001*</td>
<td>0.015 ± 0.002</td>
</tr>
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</table>

Values are means ± SE; n = 7, 8, and 8 for juvenile, mature, and middle-aged rats, respectively. ABP, arterial blood pressure; HR, heart rate; CBF, carotid blood flow; CVC, carotid vascular conductance. *P < 0.05, †P < 0.01, §P < 0.001, respectively, for difference between values recorded before and after nitro-l-arginine methyl ester (l-NAME). §P < 0.001, difference between value recorded in juvenile and mature or middle-aged rats.

Fig. 2. Cardiovascular responses evoked in juvenile, mature, and middle-aged rats by systemic infusion of adenosine. Open columns indicate control values of mean arterial pressure (ABP) in mmHg, heart rate (HR) in beats/min (bpm), carotid blood flow (CBF) in ml/min, and carotid vascular conductance (CVC) in ml·min⁻¹·mmHg⁻¹, while hatched columns indicate mean value recorded over 3-min infusion: diagonal hatching, cross hatching, and horizontal hatching indicates juvenile, mature, and middle-aged as shown below columns. In each case, values are shown as means ± SE. Difference between control value and value recorded during adenosine infusion: ***P < 0.001, **P < 0.01, *P < 0.05. §Difference between groups for changes evoked by adenosine: P < 0.05.
**Effects of l-NAME**

In all age groups, l-NAME at 10 mg/kg induced an increase in ABP coupled with a reduction in HR, CVC, and CBF (Table 1). In absolute terms, any differences between the effects of l-NAME on the cardiovascular variables in the three groups did not reach statistical significance. Because there were differences between the groups for the absolute value of HR and because baseline CBF and CVC tended to be higher in the larger, mature and middle-aged groups than in the juvenile group, we also calculated the effects of l-NAME as a percentage change from the baseline. There was no difference between the groups for the percentage increase in ABP, but the percentage decrease in HR was significantly greater in the middle-aged than juvenile rats, while the percentage decrease in CBF and CVC was greater in the mature and middle-aged rats than in the juvenile rats (Fig. 3).

After l-NAME (10 mg/kg), adenosine infusion induced very similar changes in juvenile rats as seen before l-NAME: ABP and HR fell while CVC increased with no change in CBF (Fig. 4). By contrast, in mature rats, the fall in ABP and HR evoked by adenosine did not change, but the increase in CVC was smaller after l-NAME than before; CBF increased during adenosine infusion as it had before l-NAME (Fig. 4). On the other hand, in the middle-aged rats, the fall in ABP and HR and the increase in CVC induced by adenosine infusion after l-NAME were not different from those recorded before l-NAME, but now, adenosine evoked a significant, substantial increase in CBF (Fig. 4).

**Effects of Higher Doses of l-NAME**

These groups of experiments were performed to establish whether doses of l-NAME > 10 mg/kg would produce even greater reductions in the adenosine-induced increases in CVC in the mature rats. l-NAME at 20 and 30 mg/kg had no greater effect on the baseline levels of the cardiovascular variables than l-NAME at 10 mg/kg (Fig. 5) and no greater effect on the adenosine-evoked fall in ABP. However, the adenosine-evoked increase in CVC was smaller after l-NAME at 20 and 30 mg/kg, such that the increase in CBF induced by adenosine was smaller than before l-NAME (Fig. 5).

**DISCUSSION**

In the present study, we have shown that adenosine can release NO from the endothelial surface of the common carotid artery of the rat in vitro and that in vivo, systemic infusion of adenosine in rats can induce vasodilation in the circulation served by the common carotid artery. Importantly, the magnitude of these responses varied between juvenile, mature, and middle-aged rats. Moreover, it was only in the mature rats that inhibition of NOS decreased the magnitude of the adenosine-induced carotid vasodilation. In the discussion below, we have considered the new information these results provide on the effects of aging on vascular responsiveness, in the context of the limitations of the techniques we used.

**In Vitro Studies**

The selectivity and sensitivity of the NO sensor used in the present study has been shown previously. The sensor does not respond to adenosine per se in the range of concentrations used in the present study, to various pharmacological agents, or to Krebs solution (11, 29). Further, when the rat aorta was prepared in exactly the same way as the carotid artery in the present study, adenosine-evoked NO release was attenuated by application of adenosine A1 or A2A receptor antagonists and abolished by removal of the endothelium or l-NAME (see 27). Similarly, in the present study, l-NAME abolished NO release evoked by adenosine in the carotid artery. The range of sensitivity values we calculated for the NO sensors and membranes used in the present study was comparable to those reported before, and we confirmed that the technique we adopted of adding adenosine to the tissue bath did not release NO by shear stress (see 11, 29). Thus it is reasonable to conclude that in the present study graded concentrations of adenosine released graded concentrations of newly synthesized NO from the endothelial surface of the carotid artery.

The range of adenosine concentrations used in the present study to evoke NO was high (10⁻⁴ to 5 × 10⁻³ M) but was comparable to the range we used on the rat aorta (29). In that study, we showed that the reason why such high concentrations were required reflects the avid mechanisms that are present, principally in the endothelium, for removal of adenosine. Thus, when the transport mechanism for adenosine and adenosine deaminase was blocked, the dose-response curve for adenosine in the aorta was shifted leftward by several orders of magnitude to 10⁻⁹–10⁻⁸ M (29). Similarly, in human and porcine coronary artery endothelial cells, the NO output evoked by a standard concentration of 10⁻⁴ M adenosine was greatly accentuated when adenosine deaminase was inhibited (21). These findings are entirely consistent with many reports in the literature that the adenosine concentrations that must be applied exogenously to produce arterial vasodilation, particularly when applied intraluminally, are far greater than those measured in vivo, and with evidence that inhibition of adenosine transport...
or deaminase substantially enhances and prolongs the dilator response to exogenous adenosine (see 25, 29 for discussion).

In rat aorta, the dose-response curve to adenosine in the absence of inhibitors reached a plateau at $10^{-3}$ M (29), whereas in the present study, a plateau was not reached in carotid arteries taken from any age group of rats at $5 \times 10^{-3}$ M, the highest concentration tested. Thus it seems that either the removal mechanisms for adenosine are stronger and/or the sensitivity of the adenosine receptor-NO signaling pathways are weaker in carotid arteries than aorta. Clearly, it is impossible to deduce whether the differences between age groups in the magnitude of adenosine-evoked NO release reflected age-related differences in adenosine removal and/or in adenosine-receptor signaling. However, as the ability of adenosine to cause relaxation of the internal carotid artery was much greater in mature pigs than newborn piglets, and as the relative potencies of adenosine analogs indicated this reflected an increase in the effectiveness of the $A_2A$ and $A_2B$ receptors (20), it would be interesting to establish whether the ability of the $A_2$ receptor subtypes to stimulate NO release from common carotid artery changes with age.

Whether or not this is the case, we can state that under similar conditions, adenosine caused greater release of NO from carotid arteries of mature than juvenile rats, in which NO release was minimal, and that adenosine-evoked NO release was smaller from carotid arteries of middle-aged than mature rats. These findings are consistent with, and may indicate explanation for, reports that basilar and internal carotid arteries of rabbit and pig showed much greater dilator sensitivity to adenosine in mature than young or newborn animals (20, 38) and that endothelium-dependent vasodilation of cerebral arteries was blunted in old rats of 22–24 mo relative to those of 6–8 mo (22). Our results suggest that in arteries that supply the brain, the ability of the endothelium to release NO, at least in response to adenosine, increases from youth to maturity but begins to decrease again well before old age, i.e., between 10–12 and 42–44 wk, corresponding to mature and middle-age rats, respectively.

**In Vivo Studies**

In the present study, we recorded blood flow from the common carotid artery as an index of gross cerebral blood flow, without attempting to restrict blood flow to the extracranial tissues by ligating the external carotid artery, as we and others have done in previous studies (see 36, 37). The reason for this was our concern that attempting such ligation in the smaller, juvenile rats risked damaging the carotid baroreceptors and chemoreceptors and/or the carotid sinus nerve. Moreover, in our previous study (36), we had shown that the common carotid artery still supplied the eye, muzzle, and roof of the mouth even after ligating the external carotid artery, consistent with much evidence of significant communication between extracranial structures and the internal carotid artery through anastomoses (13). Thus, while it is a limitation that we did not isolate the cerebral circulation, our recordings were predominantly of gross cerebral blood flow and included the same intracranial and extracranial structures in the three age groups of rats.

![Fig. 4. Cardiovascular responses evoked in juvenile, mature, and middle-aged rats by systemic infusion of adenosine after L-NAME at 10 mg/kg. Gray columns indicate control values after L-NAME. Otherwise hatched columns and asterisks are as indicated in Fig. 2. ††Difference between change evoked by adenosine in this age group before and after L-NAME at 10 mg/kg.](http://jap.physiology.org/)
It should also be noted that gross cerebral blood flow is not only regulated by cerebral microvascular resistance but also by the large extracranial arteries, including the carotid, vertebral, and basilar arteries (6, 12). For example, Heistad et al. (12) concluded that variation in the diameter of these large arteries contributed to the regulation of cerebral blood flow when challenged by hypotensive or hypertensive challenges, while Paulson and Waldemar (26) argued that dilator responses of large extracranial arteries evoked by a fall in perfusion pressure modifies the behavior of downstream intracranial arteries. The site of our recording on the carotid artery therefore means that changes in the diameter of the carotid artery distal to the recording site and of the large arteries it supplies caused directly by adenosine, or as a myogenic response to the adenosine-induced fall in systemic ABP, contributed to the recorded CBF and to the change in computed CVC. Thus, within the limitation noted above, we can make a reasonable assessment of pressure autoregulation of the carotid circulation that included gross cerebral blood flow in the three groups of rats.

Considering the responses evoked by systemic infusion of adenosine, in middle-aged rats, the dose required to induce a fall in ABP to ~60 mmHg, the lower end of the autoregulatory range for cerebral blood flow (27), was only about one-fifth that required in the juvenile and mature rats, even though baseline ABP tended to be higher in the middle-aged rats. Thus the middle-aged rats were far more sensitive to the depressor effect of adenosine. This might reflect a greater sensitivity of the adenosine receptor-dilator pathway in the systemic vasculature of middle-aged rats. However, it may also reflect differences in the structure of systemic vessels in middle-aged relative to juvenile and mature rats, for the collagen content of arterial vessels of the rat decreases with age, while the collagen content increases (8). In addition, age-related changes in the baroreceptor reflex may play a role, for the vascular component of the baroreceptor reflex becomes more sluggish with age while the cardiac component is attenuated (8). Thus the vasodilation and bradycardia evoked by adenosine that led to the fall in ABP may have been less effectively opposed by the baroreceptor reflex in the middle-aged than in other age groups. Resolving this issue was beyond the scope of this study.

Nevertheless, when ABP was reduced to the same level by adenosine in the three age groups of rats, there was, apparently, effective pressure autoregulation of CBF, in that mean CBF remained constant in the juvenile and middle-aged rats and even increased in the mature rats. However, it should be noted that CBF fell in half of the middle-aged group, suggesting that cerebral autoregulation, or at least autoregulation of the carotid circulation, to depressor responses is limited in middle age (see 19 and below). Moreover, as CBF increased in mature rats in the face of a fall in ABP, it can be deduced they showed active dilatation of carotid circulation in response to adenosine, over and above any autoregulatory dilatation to the depressor response.

Clearly, further experiments will be required to establish the extent to which these age-dependent differences in the behavior of the gross carotid circulation can be explained by age-dependent changes in the behavior of extra- and intracranial vessels, or of extra- and intracerebral vessels.

Roles of NO

Judging from the effects of NOS inhibition with L-NAME on baselines, tonically synthesized NO made similar contributions to the resting level of ABP in juvenile, mature, and middle-
aged rats. However, the percentage decrease in baseline HR was much greater in the middle-aged than juvenile rats. Bradycardia induced by NOS inhibition has been attributed to removal of a direct inhibitory effect of NO on cardiac vagal neurons and on the vagal component of the baroreceptor reflex (41). Thus the present results suggest the inhibitory influence of NO on cardiac vagal control becomes progressively greater through maturity to middle age.

Importantly, the very fact that baseline CBF fell after NOS inhibition in all three groups of rats, the percentage decreases in baseline CVC and CBF being much greater in the mature and middle-aged rats, demonstrates that the decrease in CVC did not simply reflect a myogenic vasoconstrictor response to the rise in ABP. Rather, it more likely reflected removal of the tonic dilator influence of NO synthesized in the carotid circulation by endothelial NOS (e.g., 28, 39). Additionally, since L-NAME at doses > 5 mg/kg can cross the blood-brain barrier and inhibit neuronal NOS (nNOS) (17), it may have acted partly by inhibiting a tonic influence of NO generated by nNOS on cerebral circulation (39). Irrespective, the present study is the first to suggest that the tonic dilator influence of NO on the carotid circulation that mainly supplies the brain, is substantially greater in mature and middle-aged rats than in juvenile rats.

Seen in this context, the finding that in juvenile rats, L-NAME had no effect on the adenosine-evoked depressor response or associated increase in CVC, and that CBF still remained constant, is not surprising. It seems that in juvenile rats, NO was not essential for the carotid autoregulatory dilator response to a fall in ABP to 60–70 mmHg. There is also no reason to suggest that NO actively contributed to any increase in CVC that was directly evoked by adenosine. In fact, these results are consistent with the finding that adenosine evoked minimal release of NO from carotid artery in juvenile rats and suggest that this may also be true of vasculature supplied by the carotid artery.

By contrast, in mature rats, L-NAME at 10 mg/kg reduced the adenosine-evoked increase in CVC, and L-NAME at 30 mg/kg further reduced in the increase in CVC and caused a concomitant reduction in the associated increase in CBF. This suggests that NO contributes to the increase in CVC evoked by adenosine in mature rats, a proposal that is fully consistent with the finding that adenosine evoked substantial release of NO from the carotid artery of mature rats. That L-NAME at 10 mg/kg increased baseline ABP and reduced the increase in CVC evoked by adenosine accords with evidence that this dose inhibits tonic and agonist-stimulated NO synthesis (10, 33). However, the finding that higher doses of L-NAME had greater effects on the adenosine-evoked increase in CVC without affecting baseline ABP suggests that doses of >10 mg/kg L-NAME may be required to block adenosine-stimulated NO synthesis in carotid circulation and, possibly, autoregulatory dilation to an adenosine-induced fall in ABP (18).

We acknowledge it is possible that the adenosine-evoked increase in CVC was attenuated because the increased level of vasoconstrictor tone and/or the reduced level of cGMP in vascular smooth muscle after NOS inhibition (4) caused a general decrease in responses evoked by dilator substances. This should be investigated in future studies by testing responses evoked in carotid circulation by adenosine and other dilator agonists while an NO donor or cell-permeant cGMP is infused after NOS inhibition, so as to restore baseline levels of ABP and CVC (see 5). However, we note that the present findings that exogenous adenosine caused greater release of NO from the carotid artery of mature than juvenile rats and evoked active, NO-dependent carotid vasodilation in mature but not juvenile rats, is consistent with our previous evidence that a selective adenosine receptor antagonist attenuated cerebral vasodilation induced by systemic hypoxia in mature but not juvenile rats (see 36, 37). Taking these results together, it seems reasonable to propose that the physiological role of adenosine as a local vasodilator in the circulation supplied by the carotid artery increases between the juvenile and sexually mature stages of life.

Finally, in the middle-aged rats, L-NAME at 10 mg/kg had little apparent effect on the magnitude of the depressor response evoked by adenosine and the concomitant increase in CVC, but allowed CBF to increase, so contrasting with the lack of effect of adenosine on CBF before L-NAME. The simplest explanation for this finding is that the increase in baseline ABP induced by L-NAME brought the level of ABP reached during the adenosine-evoked depressor response back into the autoregulatory range for all the middle-aged rats (see above): the ABP fell only to ~95 mmHg rather than to ~60 mmHg. This proposal agrees with the report that the lower limit of the cerebral autoregulatory range increased from 60–70 mmHg in rats of 8 wk of age to ~90–100 mmHg at 56 wk of age (19, cf 27), a finding that was attributed to a decrease in the compliance of intra- and extracranial vessels with age (see 8, 19). Indeed, it seems that in middle-aged rats, this structural change in the vasculature means that the tonic vasodilator effect of NO on ABP and CVC effectively limits their ability to autoregulate CBF in the face of a fall in ABP.

The active carotid vasodilation that occurred in response to adenosine after L-NAME that allowed CBF to increase despite the fall in ABP may have been mediated by an increase in NO synthesis that persisted after L-NAME at 10 mg/kg. This proposal is consistent with the ability of adenosine to release NO from carotid arteries of middle-aged rats (see above) and could be tested by using a higher dose of L-NAME before and after manipulating baseline levels of ABP and CVC with NO donor or cGMP (see above).

In summary, the present study on the rat showed that responses evoked by adenosine in the carotid artery in vitro and by systemic infusion of adenosine in the carotid circulation, which mainly supplies the brain, change from the juvenile to middle-aged stages of life. Notably, the results suggest the following. 1) Pressure autoregulation of the carotid circulation to adenosine-evoked depressor responses is effective in juvenile and mature rats, but limited in middle-aged rats. 2) Adenosine can evoke substantial active vasodilation in carotid circulation of mature but not juvenile rats, and this vasodilation is NO dependent. 3) In middle-aged rats, the ability of adenosine to evoke active vasodilation in carotid circulation is restored when baseline ABP is raised by partial NOS inhibition; this may reflect the age-dependent increase in the lower limit of cerebral autoregulation. Given published evidence that locally released adenosine contributes to the cerebral vasodilator response to hypoxia and to pressure autoregulation, the present findings suggest that the contribution of adenosine and NO to these responses increases from youth to maturity but is altered
by middle age even in the rat, a species that rarely develops atherosclerosis (8).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES
