Sensitivity of norepinephrine-evoked vasoconstriction to pertussis toxin in the old rat

ROBERT, Alain, NGUYEN N. P. TRAN, PHILIPPE GIUMMELLY, JEFFREY ATKINSON, and CHRISTINE CAPDEVILLE-ATKINSON. Sensitivity of norepinephrine-evoked vasoconstriction to pertussis toxin in the old rat. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R1604–R1612, 1998.—In male Wistar rats, the in vitro vasoconstrictor response of the perfused tail artery elicited by norepinephrine or serotonin decreased with age (24 mo old vs. 3 mo old), whereas the fluorescent signal (fura 2) produced by intracellular serotonin decreased with age (24 mo old vs. 3 mo old). The response of the perfused tail artery elicited by norepinephrine (43): R1604–R1612, Ca2+ whether the G protein-modulated amplification of the vascular effector response by activation of the α-adrenoceptor antagonist will restore G protein function. This hypothesis was tested by repeating the experiments described in the previous paragraph in rats treated for 1 mo with the α-adrenoceptor antagonist nercigolone. We decided on nercigolone because this drug is not only an α-adrenoceptor antagonist but is also widely used in the treatment of cerebral and extracerebral arteriopathies of the elderly. Furthermore, the vascular effects of chronic nercigolone treatment are little known.

METHODS AND MATERIALS

Animals

Male normotensive 3 (young)-, 12 (adult)-, and 24 (senescent)-mo-old Wistar rats (body weights 427 ± 13, 576 ± 10, and 624 ± 11 g, respectively, n = 30 per age group; Iffa Credo, L’Arbresle, France) were used. Subgroups of rats aged 2, 11, or 23 mo were treated for 1 mo with daily subcutaneous injections of solvent (0.15 M NaCl, 1 ml/kg) or nercigolone at a nonhypotensive dose (2 mg/kg) (28). Nercigolone is a selective α1-adrenergic antagonist (2) that has the same potency as prazosin in the rat; nercigolone-induced vasoconstriction in the rat tail artery is primarily mediated via an α1-adrenoceptor (3).

The day-night cycle was fixed as follows: lights out from 8 PM until 8 AM. Rats were given a standard rodent diet (A04; Usine d’Alimentation Rationnelle, Villemoisson sur Orge, France) and water ad libitum.

[Ca2+]1-Vasoconstriction Coupling

The technique used has been described in detail previously (4, 5). Under anesthesia (pentobarbital sodium, 60 mg/kg ip) and additional injections of solvent or nercigolone to balance the 24-h loss, a 1-cm-long segment of the proximal portion of the tail artery was dissected out and placed in cold physiological salt solution (PSS; in mM: 140 NaCl, 5 KCl, 1.5 CaCl2, 1 MgCl2, 10 HEPES, and 6 glucose; pH 7.4).

The endothelium was removed by passing a stainless steel wire of suitable diameter through the lumen. Segments were placed in the cuvette of a spectrofluorometer (RF-5000; Shimadzu, Kyoto, Japan) and were perfused with oxygenated PSS at a constant flow rate of 1.5 ml/min at 37°C. Vasoconstriction was evaluated from the changes in perfusion pressure (mm Hg).
Segments were perfusion loaded with the Ca\(^{2+}\)-sensitive dye fura 2-AM (5 \(\mu\)M, 90 min, followed by a 20-min washout; Molecular Probes, Eugene, OR) (4, 5). Ca\(^{2+}\) mobilization was measured in parallel with vasoconstriction from the increase in fluorescence (arbitrary units) at 340 nm (F\(_{340}\)) and the decrease in fluorescence at 380 nm (F\(_{380}\)). Both values were corrected by subtraction of the background autofluorescence (AF; F\(_{340/380}\) = F\(_{340}\) - AF\(_{340/380}\)). The ratio of the signals (R = F\(_{340}\)/F\(_{380}\)) was calculated. Internal calibration was performed by determining maximal (R\(_{max}\), 4 mM Ca\(^{2+}\), 10 \(\mu\)M ionomycin) and minimal (R\(_{min}\), 0 mM Ca\(^{2+}\), 10 \(\mu\)M ionomycin, 10 mM EGTA) fluorescence as described by Scanlon et al. (33).

Ca\(^{2+}\)\(^{-}\) was calculated as follows (11, 33): (Ca\(^{2+}\)\(^{-}\)) (nM) = K\(_{d}\) × [(R’ - R\(_{min}\))/(R\(_{max}\) - R')] × \(\beta\)', where \(\beta\)' is the ratio of the baseline F\(_{340}\) signals at zero and saturating Ca\(^{2+}\) and K\(_{d}\) is dissociation constant. K\(_{d}\) increases following addition of proteins to the calibration solution (19). A K\(_{d}\) value of 224 nM was used in the formula for the young and adult animals (3- and 12-mo-old rats) because the protein content of the rat tail artery does not change with maturation (5.3 \(\mu\)g/mg, 0.5 \(\mu\)g/mg, respectively, P > 0.05). The protein content of the rat tail artery increases with age (24-mo-old rats, 7.6 \(\mu\)g/mg, +33–43%, P < 0.05, vs. 3- or 12-mo-old rats; see also Ref. 36): a K\(_{d}\) value of 283 nM (increase of 26% as compared with 224 nM) was incorporated into the formula for the old rats. This value was calculated using the regression of K\(_{d}\) vs. protein given by Konishi et al. (19). This problem has been discussed by Capdeville-Atkinson et al. (5) and Chen and Rembold (6).

The existence of calcium-insensitive, fluorescent forms of fura 2 was checked by measuring fluorescence in the presence of MnCl\(_{2}\) (1 mM) (33).

The Ca\(^{2+}\)-sensitivity of contraction was calculated as increase in perfusion pressure/increase in [Ca\(^{2+}\)] (mmHg/nM).

Specific Protocols

Norepinephrine, serotonin, and calcium. Fura 2-AM-loaded segments from 3- and 24-mo-old Wistar rats were perfused with norepinephrine or serotonin (2 min, with 5-min interval between each stimulation). Noncumulative dose-response curves (0.1 to 30 \(\mu\)M) were constructed (n = 10 per group). Other segments were perfused with a calcium-free, high-K\(^{+}\), depolarizing solution (80 mM; osmotic pressure maintained constant by replacement of NaCl) for 4 min before and throughout the construction of a noncumulative dose-response curve for calcium chloride (1, 3, and 10 mM, 3 min each, 5 min of calcium-free, 80 mM KCl between each, n = 10 per group).

G protein inhibition. Contractions evoked by norepinephrine (3 \(\mu\)M for 2 min) and high K\(^{+}\) (4 min high-K\(^{+}\), calcium-free PSS, then 2 min high-K\(^{+}\) PSS plus 3 mM CaCl\(_{2}\)) were measured in the presence of pertussin toxin (100 ng/ml) (30). Pertussis toxin was perfused from 10 min before fura 2 loading up to the end of the washout of fura 2-AM. These experiments were performed in arterial segments from 3-, 12-, or 24-mo-old Wistar rats treated with nicergoline or solvent (n = 10 per treatment and age group). Responses were compared with those obtained in arteries, which followed the same protocol except that pertussis toxin was not perfused.

Plasma norepinephrine levels. In separate subgroups of 3-, 12-, or 24-mo-old rats treated with nicergoline or solvent (n = 10 per treatment and age group), a 1-ml blood sample was taken by aortic puncture under pentobarbital sodium anesthesia (in an ice-cold tube containing 10 U/ml heparin) for determination of plasma norepinephrine levels as previously described (36). It should be noted that catecholamine values are not baseline but pentobarbital sodium-stimulated values.

Statistics

Results are expressed as means ± SE; n = number of animals. ANOVA was performed, and comparisons between means were made by the Bonferroni test. The variance of any three-way interaction was included in the error mean square. Linear-regression ANOVA was performed, and results were expressed as a = intercept and b = slope. Regression ANOVA t values are given for a and b values. Group size was 10 animals throughout.

Individual dose-response curves were fitted by least-squares analysis to a sigmoid model: y = c/1 + exp[(a - x)/b] + d, where y = response (mmHg or nM), x = log[a0/vasoconstrictor], a = log[10](ED\(_{50}\)), b = slope, c = response at dose = infinity, and d = response at dose = 0.

Constants a, b, c, and d were treated as independent, parametric variables, and averages of the individual values of the animals that constituted a given group (n = 10) were calculated.

The average values of the constants of the standardized sigmoid model were used to draw the lines joining the points in the various graphs. Given the log-normal distribution of equieffective doses of agonists (9), comparisons between dose-response curves for different groups were made by calculating the shift at the same, approximately midrange, value using log[a0(1)] values.

Chemicals and Reagents

Chemicals and reagents were bought from Sigma Chemical, St. Louis, MO. Nicergoline was kindly donated by SPECIA Laboratories, Paris, France.

RESULTS

Baseline Values

Perfusion pressure was lower in tail arteries from senescent rats (322 mmHg, P < 0.05) than in those from young rats (392 mmHg). [Ca\(^{2+}\)]\(^{+}\), was higher in senescent rats, 1.196 nM, P < 0.05) than in young (604 nM) rats (n = 30 per age group). The increase in [Ca\(^{2+}\)]\(^{+}\), with age was not a fluorescence artifact. Although autofluorescence and fluorescence in the presence of MnCl\(_{2}\) increased slightly with age, loading intensity (F\(_{360}\)/AF\(_{360}\)) and calibration factors (R\(_{max}\), R\(_{min}\), and \(\beta\)') were unaffected (Table 1). The Ca\(^{2+}\)-sensitivity of baseline tone was lower in senescent (0.27 ± 0.01 mmHg/nM, P < 0.05) than in young (0.65 ± 0.03 mmHg/nM) rats. Nicergoline pretreatment did not modify baseline values of perfusion pressure or [Ca\(^{2+}\)]\(^{+}\), (data not shown).

Responses to Norepinephrine and Serotonin

Ca\(^{2+}\)-mobilization following agonists was biphasic; there was a monophasic increase in perfusion pressure (for typical recordings see Refs. 4 and 5).

Increases in perfusion pressure elicited by norepinephrine or serotonin were greater in younger rats (Fig. 1). At ED\(_{100}\) (mmHg), the shift for norepinephrine was fivefold and for serotonin was threefold (both P < 0.05). Increases in [Ca\(^{2+}\)]\(^{+}\), were greater in senescent rats. At ED\(_{35}\) nM, the shift for norepinephrine was fourfold and for serotonin was fivefold (both P < 0.05).
The Ca\textsuperscript{2+} sensitivity of contraction was proportional to dose and similar for the two agonists. Ca\textsuperscript{2+} sensitivity was greater in younger rats (Fig. 1).

Responses to Calcium

Vasoconstriction and [Ca\textsuperscript{2+}] responses were monophasic (for typical recordings see Refs. 4 and 5). Vasoconstriction and Ca\textsuperscript{2+} mobilization were similar in young and senescent rats (Fig. 2).

In young rats, Ca\textsuperscript{2+} sensitivity of contraction after high K\textsuperscript{+} was an order of magnitude less than that produced by receptor activation (compare Figs. 1 and 2). The Ca\textsuperscript{2+} sensitivity of contraction was slightly but significantly higher in senescent animals at 3 mM CaCl\textsubscript{2} but not at 1 or 10 mM CaCl\textsubscript{2} (Fig. 2).

Plasma Norepinephrine

There was a 69% increase in plasma norepinephrine level in senescent rats (Fig. 3). Nicergoline treatment had no effect on plasma norepinephrine level.

G Protein Inhibition

Within each age group, vasoconstrictor responses to norepinephrine and to high K\textsuperscript{+} before fura 2 loading were similar in control and the pertussis toxin groups (Table 2).

Responses elicited by norepinephrine and high K\textsuperscript{+} before pertussis toxin were similar to those obtained in the corresponding age groups above (compare Figs. 4 and 5 with Figs. 1 and 2). Pertussis toxin lowered the vasoconstrictor response to norepinephrine in young and adult rats but not in senescent rats (Fig. 4). Pertussis toxin had no effect on fura 2 loading or calibration (Table 1) or [Ca\textsuperscript{2+}] (Figs. 4 and 5). Pertussis toxin did not modify vasoconstriction or Ca\textsuperscript{2+} mobilization produced by high K\textsuperscript{+} (Fig. 5).

Nicergoline Pretreatment

Chronic pretreatment with nicergoline had no effect on plasma norepinephrine levels (Fig. 3) but restored the sensitivity to pertussis toxin of vasoconstriction evoked by receptor activation in senescent rats (Fig. 4). There was a slight but significant decrease in the Ca\textsuperscript{2+} sensitivity of contraction produced by receptor activation in young (2.33 ± 0.27 mmHg/nM control, P < 0.05) and adult (2.43 ± 0.27 mmHg/nM control, P < 0.05) rats.

Nicergoline pretreatment had no effect on fura 2 loading or calibration (Table 1), Ca\textsuperscript{2+} mobilization (Figs. 4 and 5), or responses to high K\textsuperscript{+} (Fig. 5).

DISCUSSION

Our results show that the Ca\textsuperscript{2+} sensitivity of receptor-activated contraction is reduced with age. We suggest that this decrease involves a diminution of pertussis toxin-sensitive G\textsubscript{i}o levels or activity.

Before discussing possible mechanisms for the way in which such age-related changes come about, we will discuss whether methodological artifacts do not con-
found our hypothesis. The Wistar rat we used remains normotensive throughout its lifespan (22), and at the dose we used nicergoline has little effect on blood pressure (Ref. 2 and unpublished data). Thus we are dealing with vascular aging without the complications of hypertension and its treatment. The body weight of senescent rats was 146% that of young rats. We do not think, however, that these senescent animals are obese and diabetic because resting glucose levels were not elevated (results not shown). Furthermore, although naturally occurring diabetes mellitus has been reported in rhesus monkeys, it has not been reported in Wistar rats.

Structural changes can probably be eliminated because neither age nor nicergoline pretreatment modified the lumen diameter or wall-to-lumen ratio (unpublished results). Fluorescence artifacts can also be eliminated. Although autofluorescence increased with age, possibly because of an increase in the amount of collagen, a highly fluorescent protein, in the wall, age had no effect on fura 2 loading or internal calibration (neither did nicergoline or pertussis toxin).

Although changes in $[\text{Ca}^{2+}]_{i}$ occur fairly rapidly, arteries can require several minutes to achieve a steady-state mechanical response. Thus the question arises as to whether the apparent changes in $[\text{Ca}^{2+}]_{i}$ sensitivity observed could in fact be due to changes in the kinetics of the mechanical responses. We believe that this is unlikely because perfusing norepinephrine for 30 min in arteries from adult Wistar rats produced a maximal plateau response that was no greater than that produced by 2 min of perfusion (data not shown).

The fact that pertussis toxin and nicergoline have no effect on $[\text{Ca}^{2+}]_{i}$ suggests the existence of an additional


α-adrenoceptor that is not coupled to Gq and hence to Ca\(^{2+}\) mobilization but is coupled via a different G protein to some Ca\(^{2+}\)-independent pathway. We have recently demonstrated the existence of pertussis toxin-sensitive Gi/o proteins and their in vitro ribosylation by pertussis toxin in the rat tail artery using a combination of molecular biology and pharmacometrics (31, 35). Our working hypothesis is that α-adrenoceptors in the rat tail artery are linked to intracellular contractile pathways via not only Gq but also pertussis toxin-sensitive Gi/o, that Gi/o is responsible for Ca\(^{2+}\) sensitization, that aging decreases Gi/o levels or activity, and that aging causes this effect because of increases in norepinephrine levels.

Several authors have raised the possibility that receptors linked to contractions couple not only to Gq but also to pertussis toxin-sensitive Gi/o. In vivo pertussis toxin lowers vascular resistance (13). Pertussis toxin has been shown to diminish the contractions to norepinephrine (1). The latter authors concluded that the action of pertussis toxin on norepinephrine-induced contraction of blood vessels was due to inhibition of G proteins coupled to α1-adrenoceptors. The α1-adrenoceptor agonist phenylephrine stimulates guanosine 5’-O-(3-thiotriphosphate) (GTP\(_{\gamma}\)S) binding to Gi in the rat aorta (15). Thus, besides the well-known α-adrenergic activation of pertussis toxin-insensitive Gq causing activation of phospholipase C and Ca\(^{2+}\), it is also possible that norepinephrine activates other G proteins, such as Gi/o. It has been shown in the rat tail artery that norepinephrine stimulates phosphoinositol breakdown (and hence presumably mobilizes Ca\(^{2+}\)) by a pertussis toxin-insensitive G protein (7, 21). G protein activation (with NaF + Al) contracts cerebral arteries, and this effect is not modified by the addition of a phospholipase C inhibitor, U-73122 (30).

![Graphs showing calcium-induced increases in perfusion pressure, Ca\(^{2+}\) mobilization, and Ca\(^{2+}\) sensitivity of contraction in tail artery segments from young (■) and senescent (●) rats; n = 10 per group.](image)

![Graphs showing plasma norepinephrine levels of male Wistar rats of various ages, treated or not treated with nicergoline (2 mg/kg, per day for 1 mo). Filled bars, nicergoline injected subcutaneously; open bars, controls. Blood samples were withdrawn under pentobarbital sodium anesthesia. *P < 0.05 senescent vs. young, ANOVA and Bonferroni test (n = 10 per group).](image)

**Table 2.** Perfusion pressure changes evoked by norepinephrine and high-K\(^{+}\) solution before fura 2 loading in the control and PTX series

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<th>Control</th>
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<td>Solvent</td>
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<td>Young</td>
<td>106 ± 28</td>
<td>124 ± 28</td>
<td>68 ± 12</td>
<td>53 ± 13</td>
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<tr>
<td>Adult</td>
<td>111 ± 28</td>
<td>146 ± 26</td>
<td>58 ± 15</td>
<td>65 ± 13</td>
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<tr>
<td>Senescent</td>
<td>66 ± 4</td>
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<td>Nicergoline pretreatment</td>
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<tr>
<td>Young</td>
<td>123 ± 22</td>
<td>103 ± 15</td>
<td>68 ± 18</td>
<td>58 ± 14</td>
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<tr>
<td>Adult</td>
<td>115 ± 21</td>
<td>100 ± 30</td>
<td>65 ± 12</td>
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<tr>
<td>Senescent</td>
<td>114 ± 16</td>
<td>106 ± 15</td>
<td>65 ± 16</td>
<td>50 ± 12</td>
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Values are means ± SE. Dose of norepinephrine was 3 μM; high-K\(^{+}\) solution contained 80 mM KCl and 3 mM CaCl\(_2\). All P values were >0.05.
In addition to the coupling of receptors to multiple G proteins, the possibility of activation of multiple receptor subtypes also exists. A subpopulation of $\alpha_2$-adrenoceptors contributes to the vasoconstrictor response of the rat tail artery to norepinephrine (25). Pertussis toxin blocks vasoconstriction produced by $\alpha_2$-adrenoceptor agonists (23, 26). It is also possible that the rat tail artery constricts following activation of a heterogeneous population of $\alpha_1$-adrenoceptors (3).

Aging was accompanied by a decrease in $[\text{Ca}^{2+}]_i$ sensitivity of agonist-induced contraction. With depolarization-induced contraction, there was a slight increase in $[\text{Ca}^{2+}]_i$ sensitivity of contraction on addition of 3 mM CaCl$_2$ in a depolarized preparation but not at 1 or 10 mM and not when arteries were depolarized in the presence of 3 mM CaCl$_2$. This result implies that the age-linked decrease in $[\text{Ca}^{2+}]_i$ sensitivity of contraction is a phenomenon related to receptors.

Several authors have shown that although pertussis toxin blocks agonist-induced vasoconstriction, it does not modify the effects of high K$^+$ (17, 29), suggesting that the agonist-induced amplification of vasoconstriction is mediated by a G$_{ip}$ protein. Receptor activation increases the Ca$^{2+}$ sensitivity of contraction through a G protein-mediated pathway (10). Furthermore, it has been shown that the high Ca$^{2+}$ sensitivity of the myosin phosphorylation pathway is modulated by Ca$^{2+}$-independent G proteins and that GTP analogs increase Ca$^{2+}$ sensitivity in smooth muscle cells by inhibition of the dephosphorylation of myosin light chain by phosphatase(s) (18, 20). Based on these observations, our working hypothesis is that the difference in Ca$^{2+}$ sensitivity
between contraction induced by high $K^+$ and that induced by agonists can be explained by the existence in the latter case of a pertussis toxin-sensitive, $G_{i/o}$ protein-linked, $Ca^{2+}$-independent amplification pathway that inhibits the dephosphorylation of myosin light chain.

The third element in the argument outlined above is that aging decreases $G_{i/o}$ levels or activity. $G_{ia}$ expression is reduced in aorta of 24-mo-old Fischer 344 rats (16). The data for this group suggest that arterial $\alpha_1$-adrenoceptors are coupled to $G_i$, $G_s$, and $G_q$ (16) and that age-related reductions in G proteins could account for alterations in vascular receptor function during aging. It has also been shown that vascular pertussis toxin labeling declines with age (24).

The fourth element is that high norepinephrine levels are the cause of the age-related decrease in $G_{i/o}$ levels or activity. Several studies have shown that plasma norepinephrine levels increase with age (8, 36, and present results). In other hyperadrenergic states such as heart failure (14) or prolonged infusion of norepinephrine (15), it has been shown that the high norepinephrine level desensitizes G proteins. Six days of intravenous perfusion of norepinephrine reduced the ability of phenylephrine to stimulate $GTP \gamma S$ binding in aorta, and the authors concluded that vascular desensitization involves reduction in the ability of $\alpha_1$-adrenoceptor agonists to activate $G_i$ (15). Infusion of norepinephrine decreases $G_{ia}$ and $G_{ia}$ mRNA levels in aorta (37).
Although there are a multitude of neurohormonal changes in aging, we suggest that the desensitization process involves primarily norepinephrine because "protection" of the receptors with nicergoline restored the sensitivity of norepinephrine-induced contraction to pertussis toxin.

In our experiments, the effect of norepinephrine does not appear to be limited to noradrenergic receptors because the Ca\(^{2+}\) sensitivity of contractions induced by serotonin was also altered. This could involve a process of heterologous desensitization (27). Several groups have shown that pertussis toxin attenuates vasoconstrictor responses to serotonin (e.g., Ref. 12).

Perspectives

Since it has been shown that age leads to a partial loss of a pertussis toxin-sensitive, G\(_{\alpha}\) protein-linked pathway that increases the Ca\(^{2+}\) sensitivity of agonist-induced contraction, future work will center on the search for the intracellular enzymes involved in this pathway. These could represent new intracellular targets for the development of drugs useful in the treatment of age-linked disorders of mechanisms regulating local tissue blood flow.

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