Journal of Biomedical Science (2007) 14:757–765
DOI 10.1007/s11373-007-9191-1

Influence of nitric oxide-mediated vasodilation on the blood pressure measured with the tail-cuff method in the rat

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Received 28 December 2006; accepted 26 June 2007
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Key words: L-NAME, systolic blood pressure, nitric oxide, tail-cuff method

Abstract

Systolic blood pressure (SBP) is frequently measured in rats by the tail cuff method, which usually comprises pulse/flow disappearance and reappearance during cuff inflation (Inf) and deflation (Def), separated by an interval between cycles (IBC). Although Def values are habitually used to estimate SBP, in 58 Wistar rats we found (Def–Inf) to be -6 ± 1 mmHg, indicating that Def < Inf in most cases. When the IBC was lengthened to 2 min, (Def–Inf) was increased to -17 ± 2 mmHg, indicating the probable accumulation of a vasodilating metabolite. This increase of (Def–Inf) was prevented by papaverine, indicating its relation to smooth muscle contractility. Adrenergic blockade did not prevent the increase of (Def–Inf), but pretreatment with L-NAME decreased it to -5 ± 2 mmHg ($p < 0.05$). Simultaneous measurement of SBP by tail-cuff method and carotid cannulation revealed that the Inf value was the most accurate estimation of intravascular SBP. We conclude that: (1) the Inf value should be taken as representative of SBP, since depending on the duration of suprasystolic compression the Def value can underestimate it, and (2) nitric oxide accumulation due to flow deprivation was the main cause of SBP underestimation by Def values.

Introduction

Despite its shortcomings, the tail-cuff method for measuring systolic blood pressure (SBP) in rats is still very useful, especially when the screening of large populations of animals and/or the follow-up of SBP over long time periods are involved [1]. Several methods of flow/pulse detection have been described in addition to the original water plethysmograph [2] or modifications thereof [3–6]: mercury-in-rubber plethysmograph [7], Doppler effect [8–11], photoelectric [12–15], impedance [16], microphonic [17–19], and piezoelectric pulse detectors [20–23], this last being the most widely employed.

With most of these methods it is possible to detect two events associated with the SBP: (1) disappearance of the pulse/flow signal during cuff inflation (Inf), and (2) its reappearance during deflation (Def).

However, in a revision of the bibliography specifically related to methodology of BP measurement, pulse/flow reappearance is the value overwhelmingly taken as representative of SBP [3, 7, 12, 24], even in papers which performed and reported both readings [25–28], but no reason was given for this election.

This would be a non-important matter if both values were equal, but in our laboratory's experience the readings obtained during the Def cycle are lower than the ones recorded during the Inf cycle. Careful inspection of the figures presented in some papers [8, 21, 25, 27] reveals the same fact. However, we found only two papers that specifically

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discussed differences between both measurements, one of them stating that the SBP values obtained during Inf and Def cycles were not statistically different [29]. In the other, however, the authors reported that the BP obtained during the Inf cycle is on the average 11 mmHg higher that the one measured during Def, and the difference was statistically significant [5]. They also found that the values during the Inf cycle are in closer agreement with the intraarterial measurements than the Def ones. These data are coincident with our own results, but the authors did not provide any hypothesis and/or pursue further experiments to explain the difference between Inf and Def values [5].

Since the information on this subject seems to be scarce and contradictory, we undertook experiments destined to find out: (1) if the BP values obtained with the tail-cuff method are coincident during the Inf and Def cycles, and (2) In case of a lack of coincidence, which of the values better represents the intra-arterial readings, and the reason for the difference. Our hypothesis was that a vasodilator accumulated in the tail during the Inf–Def interval was responsible for the lower SBP values obtained during Def.

Methods

Animals

A total of 164 Wistar rats weighing 200–250 g were used in the course of this study. The animals were housed in cages with food and water ad-libitum, complying with standard guidelines for care and use of laboratory animals.

BP measurement

For indirect SBP readings, the rats were placed in a chamber at 37 °C for 10 min, and then transferred to a standard setup with heating pad and acrylic restrainer, tail cuff and pulse sensor (Narco Biosystems, Houston, TX). The tail cuff was connected to a cylinder of compressed air through an arrangement of inlet and outlet valves that permitted Inf and Def of the cuff at a constant rate. The tail cuff pressure was continuously recorded with a solid state pressure sensor (Sensym, Honeywell Sensing & Control, Inc.). The signals from the pulse and pressure sensors were conveniently amplified and then digitized with an analog-digital board (DT16EZ, Data Translation, Inc., Marlboro, MA) mounted in a desktop computer. On-line display for controlling the procedure, and files for later processing, were obtained with an appropriate software (Labtech Notebook Pro, Laboratory Technology Corp., Wilmington, MA). The indirect BP was always recorded during the Inf and Def cycles, and the interval of time between cycles (IBC) could be changed as desired. The indirect measurements were all performed by the same person, who was kept blind about the purpose of the study.

For direct BP measurement, the rats were anesthetized with sodium pentobarbital (50 mg/kg I.P.) and placed on a heating pad. The left carotid artery was exposed and cannulated with a short microbore teflon tubing 0.75 mm O.D. (Small Parts, Inc, Miami Lakes, FL) connected with a low-volume displacement pressure transducer (P23 Gb, Gould Inc., Cleveland, OH). When the intraarterial tracing was stable, the tail cuff and pulse sensor were placed on the tail, and the indirect BP was measured at the same time. All the signals were sent to the same acquisition system (see above) so that both the indirect and direct BP tracings could be superimposed (see also Figure 1).

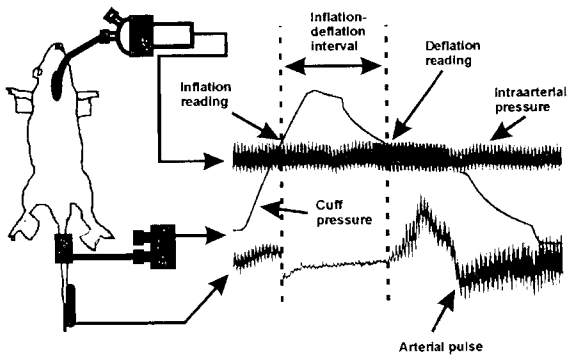


Figure 1. Left: schematic arrangement for direct and indirect measurement of BP. Right: actual recordings of simultaneous intraarterial and tail-cuff measurements, showing the variables referred to throughout the text. It should be noted that this setup was used only in 11 rats (see Figure 3); in the remaining experiments only the indirect SBP in conscious rats was used.

Histochemical NOS activity

The NADPH-diaphorase assay was used to estimate total NOS activity in tail arteries removed after anesthesia with sodium pentobarbital (50 mg/kg I.P.) under conditions that simulated as closely as possible the experimental situations compared. In the control experiments the tail was not compressed and the arteries were excised and dissected free of fat and connective tissue in a Petri dish filled with saline solution bubbled with a mixture of 5% CO_2–95% O_2. In the "ischemic" experiments the tail was compressed with the cuff maintained at 180 mmHg for 2 min. The arteries were then rapidly excised and dissected free of fat and connective tissue in a Petri dish filled with saline solution bubbled with N_2. All the vessels were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4); cryoprotected in 15% sucrose and frozen. Sections of 15 μm were cut on a cryostat, mounted on gelatin-coated glass slides, and incubated with a reaction mixture containing 1.2 mM NADPH and 0.24 mM nitro blue tetrazolium in phosphate buffer added with 0.3% Triton X-100 for 60 min at 37 °C. The NOS activities assayed by this technique are inhibited by preincubation with diphenyleneiodonium and other NOS inhibitors. A Zeiss Axiophot microscope was used for observation, absorption determination, and photography. Computerized image analysis was performed using an analyzer (Kontron Zeiss), and mean absorption values were calculated from five areas of each section and from 10 different sections. The determinations were performed under similar conditions of light, gain, offset, and magnification. The technician was kept blind about the purpose of the measurements. No reaction product was found when NADPH was omitted. In control experiments, 5 mM L-NAME was added to consider the specific NADPH-diaphorase staining due to NOS activity.

Estimation of vascular superoxide anion ($\cdot O^{2-}$) production

$\cdot O^{2-}$ production was measured using lucigenin chemiluminescence. Briefly, after preparation in the conditions described for histochemical NOS activity, the vessels were placed in a modified Krebs/Hepes buffer and allowed to equilibrate for 30 min at 37 °C. Scintillation vials containing 2 ml Krebs/Hepes buffer with 5 μM lucigenin were placed into a scintillation counter (Chameleon, Hidex Co., Finland) switched to the out-of-coincidence mode. After dark adaptation, background counts were recorded and a vascular segment was added to the vial. Scintillation counts were then recorded every 30 s for 15 min, and the respective background counts were substracted. The vessels were then dried by placing them in a 90 °C oven for 2 h, for determination of dry weight. Lucigen counts were calculated as counts $\cdot 10^3$ per min per mg dry weight.

Drugs: papaverine, propranolol, phenoxybenzamine, L-NAME and other analytical chemicals were purchased from SIGMA (St. Louis, MO).

The results were expressed as mean ± 1 S.E.M., and the differences between means were evaluated using a statistical package (Sigmastat 2.0, Jandel Scientific Software, San Rafael, CA). When only two groups were involved the Student's *t* test (paired or unpaired) was used, while for multiple groups comparisons the one-way ANOVA was employed. The particular modifications of these tests as dictated by the circumstances are indicated in Methods for each specific protocol. The correlation between variables was studied with linear regression.

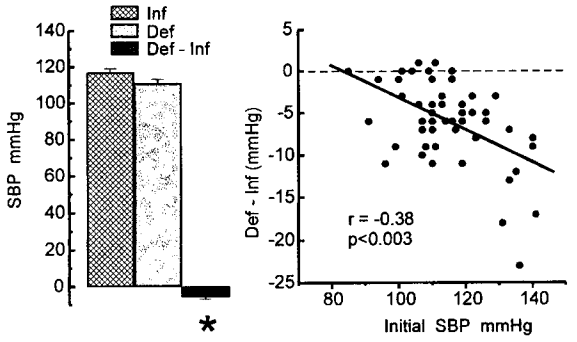


Figure 2. Left panel: SBP measured by the tail-cuff method in 58 Wistar rats, showing the Inf and Def readings and the difference between them (Def–Inf), which is significant. The Inf–Def interval was ≅30 s. Right panel: scatter graph plotting (Def–Inf) as a function of the initial SBP (i.e. the Inf reading) for each animal, showing a significant correlation between both variables. * = $p < 0.05$.

Results

Figure 2 depicts on the left panel the SBP measured during the Inf and Def cycles in 58 Wistar rats, showing that the Def readings were lower than their Inf counterparts. The average difference between Def and Inf values (Def–Inf) was slight (−6 ± 1 mmHg), although significant ($p < 0.001$, Wilcoxon Signed Rank Test). The right panel shows the dependence of the (Def–Inf) on the prevailing SBP, and it can be seen that in only seven rats the Def reading was equal to, or greater than, the Inf counterpart ($r = -0.38$, $p < 0.003$, Pearson product moment correlation). These were routine measurements of BP that had been performed recently in our laboratory, in which no particular attention was paid to the duration of the IBC. When the records were revised afterwards, the IBC had been around 30 s.

In 11 additional experiments in Wistar rats (Figure 3) we performed simultaneous measurement of BP by the tail-cuff method and by direct carotid cannulation, and we obtained similar values for the direct SBP and the Inf readings (NS, Wilcoxon Signed Rank Test). However, the Def reading was slightly but significantly lower than the directly measured SBP ($p < 0.006$, paired t test). Since these were the only experiments performed with anesthesia, we compared the indirect BP values with the ones obtained in the same rats before surgery, and we found no significant differences between them (conscious rats: 115 ± 2 mmHg, anesthetized rats: 116 ± 4 mmHg, $n = 11$, NS with the paired t test) (data not illustrated).

To evaluate the relationship between (Def–Inf) and the duration of the IBC we measured the SBP in 12 Wistar rats by the tail-cuff method in two consecutive runs (Figure 4): in the first one the IBC was less than 15 s (left bars), while in the second the IBC was increased to 2 min (middle bars). As can be seen, the lengthening of the IBC produced a significant increase of (Def–Inf) to −15 ± 2 mmHg ($p < 0.001$, paired t test).

The influence of a nonspecific smooth muscle dilator, papaverine, was evaluated in seven of the previous Wistar rats in which a third SBP measurement was performed 20 min after the administration of 75 mg/kg I.P. of papaverine and also keeping the IBC in 2 min. As can be seen in the right bars on Figure 4 the (Def–Inf) was significantly reduced by papaverine and returned to values not different from zero ($p = 0.69$, paired t test).

In another set of experiments (Figure 5) we evaluated the participation of two important routes of vascular smooth muscle control, i.e. the sympathetic innervation and the NO pathway, on the

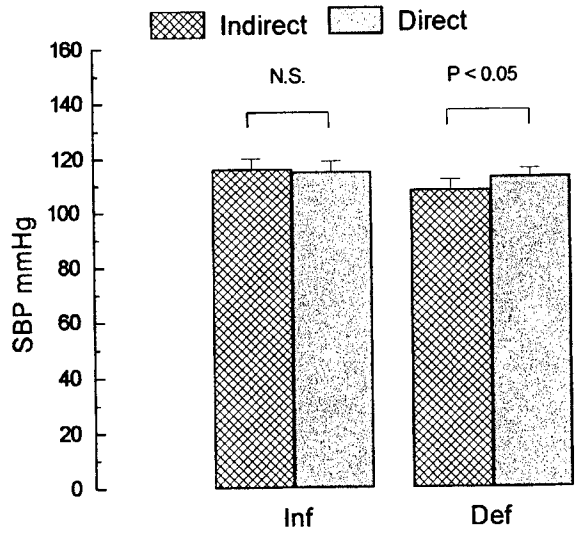


Figure 3. SBP measured simultaneously by the tail-cuff method (Inf and Def readings) and by carotid cannulation in 11 Wistar rats. The Def readings were slightly, although significantly, lower than the direct measurement; hence the Inf reading estimated the intraarterial SBP more accurately.

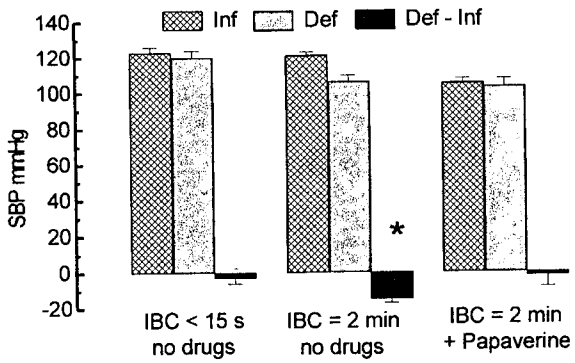


Figure 4. Effect of the length of the interval between the Inf and Def cycles (IBC) on the (Def–Inf). The bars on the left represent 12 measurements in which the IBC was less than 15 s, and the (Def–Inf) was nearly zero. The bars on the middle represent 12 measurements in which the IBC was increased to 2 min, and in this case the (Def–Inf) augmented significantly. The bars on the right represent seven measurements in which the IBC was also increased to 2 min, but with the previous administration of 75 mg/kg of papaverine i.p. ($n = 7$), and in this case the increment of the (Def–Inf) was prevented. * = $p < 0.05$ with respect to zero.

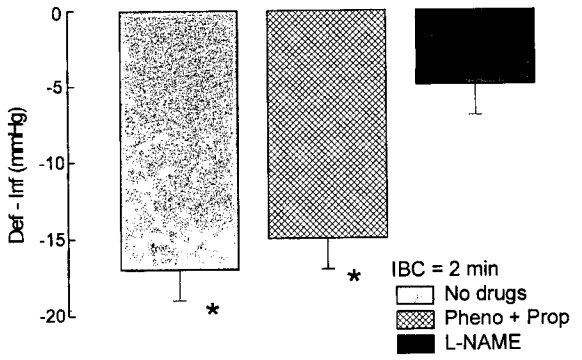


Figure 5. Experiments in which the IBC was kept deliberately at 2 min. In absence of drugs (left bar, $n = 21$) the (Def–Inf) was significant (-17 ± 2 mmHg, $p < 0.05$). Administration of phenoxybenzamine and propranolol (middle bar, $n = 8$) failed to reduce the (Def–Inf), which remained at -15 ± 2 mmHg. The right bar represents the same measurement in rats pretreated with L-NAME in the drinking water for 3 weeks ($n = 10$), and in this case the (Def–Inf) was significantly reduced to -5 ± 2 mmHg. * = $p < 0.05$ with respect to zero.

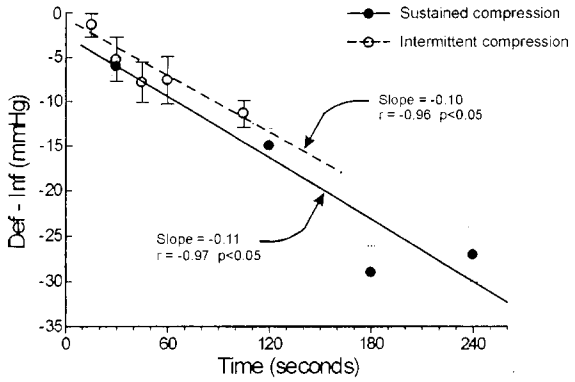


Figure 6. Experiments showing that both a continued tail compression (closed circles) and repeated short cycles of compression/decompression (open circles) can increase the (Def–Inf). The slopes of both regression lines are nearly identical. The number of experiments represented by each symbol are detailed in Methods. With the exception of the intermittent measurement at 15 s, the rest of the (Def–Inf) values were significant ($p < 0.05$), although the asterisks were omitted next to each symbol for the sake of clarity.

increase of (Def–Inf). In 17 rats with no drugs the IBC was fixed in 2 min, obtaining a significant (Def–Inf) (left bar, $p < 0.001$, paired t test). Administration of phenoxybenzamine (4 mg/kg i.p.) and propranolol (10 mg/kg i.p.) to eight of these rats produced an effective adrenergic blockade as could be ascertained by the fall in blood pressure from 103 ± 2 mmHg to 75 ± 2 mmHg (Inf readings); however, the Def cycle under adrenergic blockade produced still lower readings of 62 ± 3 mmHg. This Def–Inf difference (14 ± 2 mmHg) was not significantly different from the one obtained in absence of adrenergic blockade (-17 ± 2 mmHg). The right bar represents the same measurement in 10 rats pretreated with L-NAME 70 mg/kg per day in the drinking water for three weeks. The blockade of NO synthesis was effective as could be ascertained by an increase in BP from 111 ± 3 mmHg to 123 ± 3 mmHg at the end of the treatment (Inf readings). However, the (Def–Inf) in presence of L-NAME was decreased to -5 ± 2 mmHg, a significant reduction with respect to the control group ($p < 0.05$, one-way ANOVA).

Additional experiments were designed to compare the effect of a continued compression of the tail versus fast, repeated Infs and Defs during the same time period, and to study if there was a correlation between both types of measurement and the magnitude of the (Def–Inf). Figure 6 shows in open circles the measurements with IBC < 15 s repeated during 15, 30, 45, 60 and 105 s ($n = 14$ in each time period), and it can be seen that a significant correlation exists between the duration of the repeated measurements and the magnitude of the (Def–Inf) ($r = -0.96$, $p < 0.05$, Pearson product moment correlation). For comparison, in closer circles are shown measurements in which the tail compression was sustained during 30 s ($n = 58$), 120 s ($n = 12$), 180 s ($n = 15$) and 240 s ($n = 11$), and the correlation between IBC and (Def–Inf) is also significant ($r = -0.97$, $p < 0.05$, Pearson product moment correlation), although it should be noted that continued compression for longer than 180 s does not produce a greater (Def–Inf). With the exception of the intermittent measurement at 15 s, the rest of the (Def–Inf) values shown in Figure 6 were significant (Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunnett's Method). It is also worth mentioning that intermittent compression and decompression could not be extended for more than 120 s due to animal discomfort and unrest; while continued compression could be extended for up to 240 s without signs of animal discomfort.

In 12 additional rats we used the NADPH-diaphorase assay to estimate total NOS activity in tail arteries excised in control conditions and after 2 min. of ischemic compression. The histochemical analysis showed a significant increase in the

absorption values from 0.1210 ± 0.0042 (control experiments) to 0.1539 ± 0.0081 (ischemic arteries) ($p < 0.05$, unpaired t test).

In four additional experiments in which we measured $\cdot O^{2-}$ production using lucigenin chemiluminescence we could not detect consistent differences between control and ischemic vessels (data not shown).

Discussion

Byrom and Wilson in 1938 [7] described an apparatus consisting of a manually-inflated cuff located at the base of the tail and a distal water plethysmograph to detect the reappearance of flow during Def. Although the procedure seemed to be cumbersome and required that the animals be anesthetized, Okamoto and Aoki still used this method 25 years later in their pioneer work on the development of a strain of spontaneously hypertensive rats [25]. With this method it was inevitable to estimate BP during the Def cycle at the point where the cuff pressure was just below the systolic BP but still above the venous pressure, thus allowing the plethysmograph to detect the subsequent increase in tail volume.

In contrast, all the methods of pulse detection described thereafter, i.e. mercury-in-rubber plethysmograph [7], Doppler effect [8–11], photoelectric [12–15], impedance [16], microphonic [17–19], and piezoelectric pulse detectors [20–23], permitted to take Inf and Def readings, but the Def values were overwhelmingly used to estimate SBP. Usually no reasons were given for this election, although for example Buñag [29] indicated his preference for the Def cycle because the animal was more mobile during the Inf cycle and the possibility of an erroneous reading due to noisy signals was increased.

Taking advantage of the incorporation of digitizing techniques to our BP measuring setup we could easily retrieve several routine BP measurements made in the past and found that the Def cycle effectively yielded lower values than the Inf cycle: the (Def–Inf) was −6 ± 1 mmHg in 58 Wistar rats. Even with repeated Inf–Def cycles the Def value was decreased, leading to an erroneous estimation of SBP. This finding is in disagreement with the report of Buñag [29] who measured both cycles and found them to be not significantly different. However, in this paper as well as in others [8, 21, 25, 27] close inspection of some representative tracings does show a lower BP value during Def. On the contrary, our measurements coincide with the paper of Martinelli et al. [5] who reported a significant decrease of 11 mmHg for the Def cycle, nearly double than the one we found in normotensive rats. This difference can probably be accounted for by their method of pulse detection, a water plethysmograph, which probably implied a longer Inf–Def interval than in our experiments. When we subsequently performed simultaneous measurements of the BP by the tail-cuff method and by direct carotid cannulation we found that the Inf cycle matched the intravascular measurement; and this result is also in agreement with the findings of Martinelli et al. [5].

In analyzing the causes for this phenomenon, the simplest explanation could be that in the Inf cycle the cuff pressure has to be higher than the intravascular BP for the pulse to disappear, and the contrary holds true for the Def cycle. This in itself implies a lower estimation during Def if the intravascular BP is stable. However, even in this case the difference is likely to be too small to be detected with the current methods, and more so if the inevitable oscillations in BP over time are taken into account.

A purely physical explanation could be based on the different velocities of blood flow during the Inf and Def cycles. When the cuff is constricted, flow velocity progressively decreases to zero; but when the cuff deflates the arterial lumen is initially narrower than normal, and flow velocity is high. According to Bernoulli's theorem, if the fluid flows horizontally so that no change in gravitational potential energy occurs, then an increase in fluid velocity is associated with a decrease in fluid pressure and vice versa. The decrease of pressure against the vessel wall (i.e. the Venturi effect) could be reflected in a decrease of the cuff pressure necessary to counteract it, contributing to a difference between Def and Inf readings. This explanation would be compatible with our finding that the (Def–Inf) was significantly correlated with the prevailing BP: the greater the BP, the greater the velocity of blood flow upon reopening of the arterial lumen and the greater the (Def–Inf).

However, the foregoing explanation considers the tail artery as an entirely passive conduit, which

is not the case. When during the Def cycle the cuff pressure ceases to counteract the intravascular BP, the state of constriction of vascular smooth muscle constitutes the principal remaining resistance to flow, and it must be overcome for the arterial pulse to reappear. By decreasing the smooth muscle tone during Def, the readings obtained could appear lower than the ones obtained during Inf. This lowered smooth muscle tone could be provoked by a vasodilator accumulated during the Inf–Def interval, when the tail is rendered ischemic.

Two pieces of experimental evidence support this possibility. Firstly, the experiments in which we extended the Inf–Def interval (and thus the ischemic period) to 2 min and obtained a corresponding increase in the (Def–Inf), which went up to -17 ± 2 mmHg. Secondly, the experiments in which we employed the nonspecific smooth muscle relaxant papaverine [30] to suppress smooth muscle tone and, contrariwise, obtained a decrease of the (Def–Inf). By placing the smooth muscle in a state of relaxation beforehand, we could prevent the action of the hypothesized vasodilator.

We then set out to investigate which vasodilator could participate in this phenomenon, focusing our attention on sympathetic innervation and on the nitric oxide (NO) system. For that purpose we used BP readings with a 2 min interval between Inf and Def cycles in order to turn the (Def–Inf) well evident; the first measurement was performed in control conditions (no blockers) and the second after the specific blocker had been administered. When the sympathetic system was explored, alpha- and beta-blockade were induced simultaneously to make sure that the tail artery, usually richly innervated, did not receive any autonomic influence. Although the decrease in SBP indicated that the adrenergic blockade was successful, a significant (Def–Inf) still persisted, indicating that the sympathetic system was not relevant for the production of this phenomenon. It must be pointed out that the sympathetic blockade was probably more effective than could appear from the SBP values because the prevailing SBP had been decreased and this by itself lowers the (Def–Inf) as discussed before. Had the SBP remained stable, the (Def–Inf) would have been even greater.

Similar experiments were carried out in rats that had received L-NAME in the drinking water for the previous three weeks. In this case, the (Def–Inf) was decreased to non-significant values indicating that NO accumulation during the occlusion period was relevant in its production. It has to be noted that the blockade of NO synthesis was even more effective than could appear from the crude numbers because the prevailing SBP increased with the administration of L-NAME, and this by itself augments the (Def–Inf) as discussed before. Had the SBP remained stable, the decrease of (Def–Inf) would have been even greater.

Since these experiments provided only indirect evidence of the NO involvement in the phenomenon studied, we attempted to estimate histochemical NOS activity in the tail artery by the NADPH-diaphorase assay. These studies showed an increase of $28.5 \pm 9.9\%$ of NOS activity in the samples obtained from vessels obtained after cuff compression and preserved under hypoxic conditions. Although the histochemical measurement does not differentiate between the three NOS isoforms, it is plausible that the increased activity corresponds to eNOS, the predominant isoform in normal vascular endothelium. The inducible form (iNOS) is unlikely to be stimulated in our experimental conditions (i.e. non-atherosclerotic vessels without macrophage proliferation).

Further mechanisms of NO accumulation during the ischemic interval could be the following: (1) since there is a tonic production and release of NO by the endothelium [31], and the cuff compression precludes either the arterial inflow and the venous outflow, NO is likely to accumulate and be responsible for the vasodilation during the Def cycle; and (2) endothelial cell deformation is another cause of NO production [31], but in this case it can probably be ruled out because during the ischemic interval there is no flow and consequently no shear stress. Upon restoration of flow there will be shear stress and NO release, but its effect would be evident later on, and not when the pulse reappears.

It is well known that the ischemia produces reactive oxygen species, among which the superoxide anion ($\cdot O^{2-}$) is of critical importance [32, 33]. Many others, including H_2O_2, peroxynitrite, hypochlorous acid, the hydroxyl radical, and lipid radicals, are derived from $\cdot O^{2-}$. Both superoxide and NO are highly reactive and unstable radicals, and react very rapidly to form the major product peroxynitrite. Although peroxynitrite can produce vasodilation, this effect occurs at concentrations far in excess of the effective vasorelaxant concen-

trations of NO [34, 35] and probably was not important in our experiments. On the other hand, this superoxide/peroxynitrite production can reduce the bioavailability of NO because of oxidative inactivation [33], but this effect could not have explained the lengthening of the (Def–Inf), since it would be vasoconstricting and not vasodilating. At any rate, the lucigenin experiments failed to demonstrate a consistent difference between hypoxic and control arteries after 2 min of compression, which was probably too short a period to elicit an important $\cdot O^{2-}$ production.

To our knowledge this is the first study looking for an explanation of the difference between Inf and Def values obtained during tail cuff measurement of SBP in rats, and establishing nitric oxide accumulation during the period of flow deprivation as the main cause of this difference.

The consequences of our data for the laboratory practice are evident: the Inf value should be taken as representative of SBP, and suprasystolic compression by the tail cuff should be kept as short as possible, since the error of SBP estimation can be quite important. Surely enough, experienced operators will tend to proceed in this way and then the error inherent to the Def reading would be small or even non-existent. But if less-experienced persons and/or non-trained animals are involved, repeated attempts to measure BP will lead to prolonged tail compression and ischemia, as proved in this study, and would render Def readings that do not estimate intravascular SBP accurately.

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