Nitric oxide evokes pain at nociceptors of the paravascular tissue and veins in humans

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1. Nitric oxide (NO) evokes pain on intracutaneous application, apparently by exciting cutaneous nociceptors. To look for similarities in the responsiveness and sensitivity of other nociceptive systems to NO we determined pain intensity–concentration relations for NO applied to paravascular tissue and veins in humans.

2. NO solutions (0·4–2·0 mM) were either injected paravascularly or perfused through a vascularly isolated hand vein segment. The subjects rated pain continuously with the help of an electronically controlled visual analog scale, which made it possible to determine both the time course (latency, duration) and the intensity of NO-evoked pain.

3. Regardless of where it was applied, at concentrations above 0·7 mM NO always evoked pain of similar time course and concentration dependence. Pain increased proportionally to the concentration of applied NO, reaching subjects' tolerance maximum at four to five times the threshold concentration.

4. Pain intensity–NO concentration relations were congruent, indicating that the respective nociceptive systems are equally sensitive to NO.

5. Our observations are consistent with the hypothesis that NO is a chemical link in peripheral nociception.

Nitric oxide (NO), perhaps a chemical link in nociception (Meller & Gebhardt, 1993), evokes pain on intracutaneous injection in humans, probably by exciting cutaneous nociceptors (Holthusen & Arndt, 1994). The question, therefore, arises whether it has a similar effect on other nociceptive systems, in particular those of blood vessels (Arndt & Klement, 1991) and that of the paravascular tissue, which is believed to convey visceral pain (Lim, Liu, Guzman & Braun, 1962). Both are interesting targets for NO for the following reasons. First, bradykinin, a naturally occurring algetic and well-known liberator of NO (Sung, Arleth, Shikano & Berkowitz, 1988), elicits pseudoaffective pain behaviour on arterial injection in animals (Steranka *et al.* 1988) and also elicits pain in humans on both paravascular and intravenous application (Kindgen-Milles, Klement & Arndt, 1994). Second, both endothelium and vascular smooth muscle are the main sources of NO (Ignarro, 1989) and are structures that are in close proximity to the paravascular and venous nociceptors (Flöel & Staubesand, 1978).

In our attempt to look for similarities in the responsiveness and sensitivity of the nociceptive systems of the paravascular tissue and of veins to NO, we determined pain intensity–time curves and pain intensity–concentration relations for paravascularly and intravenously applied NO solutions in humans. We showed that NO evokes pain regardless of the site of application and that the respective nociceptor systems were equally sensitive to NO. Our observations are consistent with the hypothesis that NO is a chemical link in peripheral nociception.

METHODS

Six healthy men aged between 23 and 34 years (physicians and medical students) volunteered and consented to this study, which was approved by the Committee of Medical Ethics of the Heinrich Heine University of Düsseldorf. On two different days at least 2 weeks apart, each subject received NO solutions either paravascularly or intravenously.

Preparation of NO solutions

On each experimental day, NO (oxygen-free; Linde, Düsseldorf, Germany) was bubbled through three connected gas-tight vials after the system had been deoxygenated with argon (Linde) for 1 h. The first vial contained 10% potassium hydroxide solution for scavenging higher nitrogen oxides, the second high-grade water (Merck) for clearing potassium hydroxide aerosol, and the third, phosphate buffer (pH 7·3; 300 mosmol l^{-1}) as the solvent for preparing the stock solution, which was saturated by bubbling NO for at least 45 min.

The NO concentration in the stock solution was measured by the methaemoglobin spectrophotometry assay. NO oxidizes reduced haemoglobin within milliseconds to methaemoglobin and the conversion from haemoglobin to methaemoglobin was measured as

the difference in extinction at 401 and 411 nm (Archer, 1993). For preparing NO solutions of appropriate concentrations, aliquots of the stock solution were diluted with argon-deoxygenated, isosmolar phosphate buffer and kept under an argon atmosphere in silicon rubber-sealed vials or in Hamilton syringes.

Routes of application

Paravascular injection. NO solutions (0·05 ml) of different concentrations (0·4, 0·7, 1·0 and 2·0 mM) were injected at different sites into the paravascular tissue at the back of the hand via a steel cannula (27 g), which was advanced through a small vein to prevent NO solution from flowing back to nociceptors in the skin via the inserting channel. Intervals between injections were at least 10 min. It should be noted that haemotoma were not observed on paravascular transvenous injections.

Intravenous application. Since single injections never evoked pain, NO was continuously perfused through a vein segment at the dorsum of the hand. For blood-free perfusion, a vein segment was cannulated at distal and proximal ends between two valves (Venflon®, outer diameter 1·2–1·7 mm; Ohmeda, Helsingborg, Sweden) and vascularly isolated by fastening external occluders of foam rubber on the puncture sites (Arndt & Klement, 1991).

Pain measurement

The subjects rated pain continuously on an electronically controlled visual analog scale (VAS). A linear potentiometer, moveable from the left (0% VAS = no pain) to the right (100% VAS = maximal tolerable pain), yielded voltages proportional to pain intensity, which were recorded continuously on a Gould TA 550 Polygraph.

Programme of experimentation

The experiments always started at 09.00 h with the subjects sitting semi-recumbent in an easy chair at a thermoneutral temperature of 24 °C, with the forearm and hand placed comfortably on a cushioned armrest.

For paravascular application, NO solutions of various concentrations and the vehicle solution without NO were injected in random order into different sites on the back of the hand every 10 min in single blind experiments.

For intravenous application, the armrest was raised above heart level until hand veins had collapsed. NO solutions and the vehicle were then perfused at 10 ml min^{-1} for 4 min in random order in single blind experiments. Intervals between successive trials were at least 15 min.

Data evaluation and statistics

Pain intensity–time relations. For each subject and experiment pain intensity–time curves were reconstructed from the original recordings by determining pain intensities every 5 s for paravascularly applied NO and every 10 s for intravenously applied NO.

For detailed presentation of intensities, latencies and durations of pain responses, these variables were taken from the original recordings and listed separately as means and standard errors of the means (S.E.M.). For each variable and each subject we calculated a linear trend coefficient and compared these coefficients with zero for each variable by means of a one-sided sign test (Krauth, 1988). To avoid multiple testing, we used the P values for statistical inference only in the case of the variable pain intensity, the most important variable of pain responses (level of significance $< 0·05$). For the two tests of significance α-adjustment was performed using the Bonferroni procedure. The four other P values are given for descriptive purposes.

Pain intensity–NO concentration relations

For deriving pain intensity–NO concentration relations, maximally reached pain in each experiment was extracted from pain intensity–time curves and related to the corresponding concentration of NO.

RESULTS

NO always evoked pain at the site of application, whether it was injected or perfused. Intensity, as well as latency and duration, of NO-evoked pain depended on concentration. This is shown by the individual pain intensity–time curves in Fig. 1 and by the numerical data listed in Table 1.

On paravascular injection of various concentrations of NO (Fig. 1*A*), pain occurred at 0·7 mM and increased with increasing concentration, with the pain intensity–time relations being more consistent at higher NO concentrations. The concentration-dependent increase in intensity of pain, as the main variable of pain responses, was statistically significant and associated with an apparent trend for latencies to decrease and durations to increase with increasing NO concentrations. Neither NO below 0·7 mM nor the vehicle solution evoked pain on paravascular application (Table 1).

During intravenous perfusion of hand vein segments with various concentrations of NO (Fig. 1*B*), pain occurred as with paravascular application at 0·7 mM, but never below 0·7 mM or on perfusion of the vehicle solution. As expected, intravenously evoked pain showed a different time course. Pain occurred with latencies greater than on paravascular application and mounted, usually within 180 s, to a concentration-dependent maximum (statistically significant, Table 1), which was maintained as long as perfusion continued. Pain eventually faded in the postperfusion period but lasted longer at higher NO concentrations. Both latencies and durations depended on concentration (Table 1). Note that after the perfusion was stopped, pain faded from its maxima with a time course similar to that following cessation of paravascular application, so that the respective maxima appear to be the appropriate choice for deriving pain intensity–concentration relations.

Pain intensity–NO concentration relations

The common features of these relations (Fig. 2) are as follows. First, in every experiment pain increased proportionally to the concentration of NO, regardless of whether pain maxima were transient after injection or maintained by perfusion. Second, on both paravascular and intravenous application, pain occurred at similar concentrations, i.e. in the 25% range of VAS at 0·7 mM and, in the majority of experiments, near the tolerance maximum (VAS > 80%) at 2 mM. In two subjects,

Table 1. Intensity, latency and duration of pain on paravascularly and intravenously applied NO

	Paravascular NO			Intravenous NO		
	Pain intensity (% VAS)	Latency (s)	Duration (s)	Pain intensity (% VAS)	Latency (s)	Duration (s)
Vehicle	0	—	—	0	—	—
0·4 mM NO	0	—	—	0	—	—
0·7 mM NO	13 ± 4	37 ± 8	59 ± 12	20 ± 3	52 ± 4	283 ± 7
1·0 mM NO	38 ± 8	24 ± 3	86 ± 12	42 ± 3	42 ± 3	307 ± 4
2·0 mM NO	91 ± 4	15 ± 2	106 ± 9	76 ± 6	41 ± 3	346 ± 6
	$P < 0·05$*	$P = 0·02$	$P = 0·02$	$P < 0·05$*	$P = 0·02$	$P = 0·02$

Data are shown as means ± S.E.M; $n = 6$. Significance of differences was tested using a one-sided sign test; level of significance, $P < 0·05$.

however, pain was only about 60% VAS at 2 mM, an observation which is probably explained by the occurrence of a visible admixture of blood to the effluent at the highest concentration. At low concentrations, perfusions in these two experiments were blood free and pain ratings were similar to the other experiments. Third, and most important, the pain intensity–concentration relations are by and large congruent for both paravascular and intravenous application, as well as for the pain intensity–concentration relations of intracutaneously applied NO (Holthusen & Arndt, 1994).

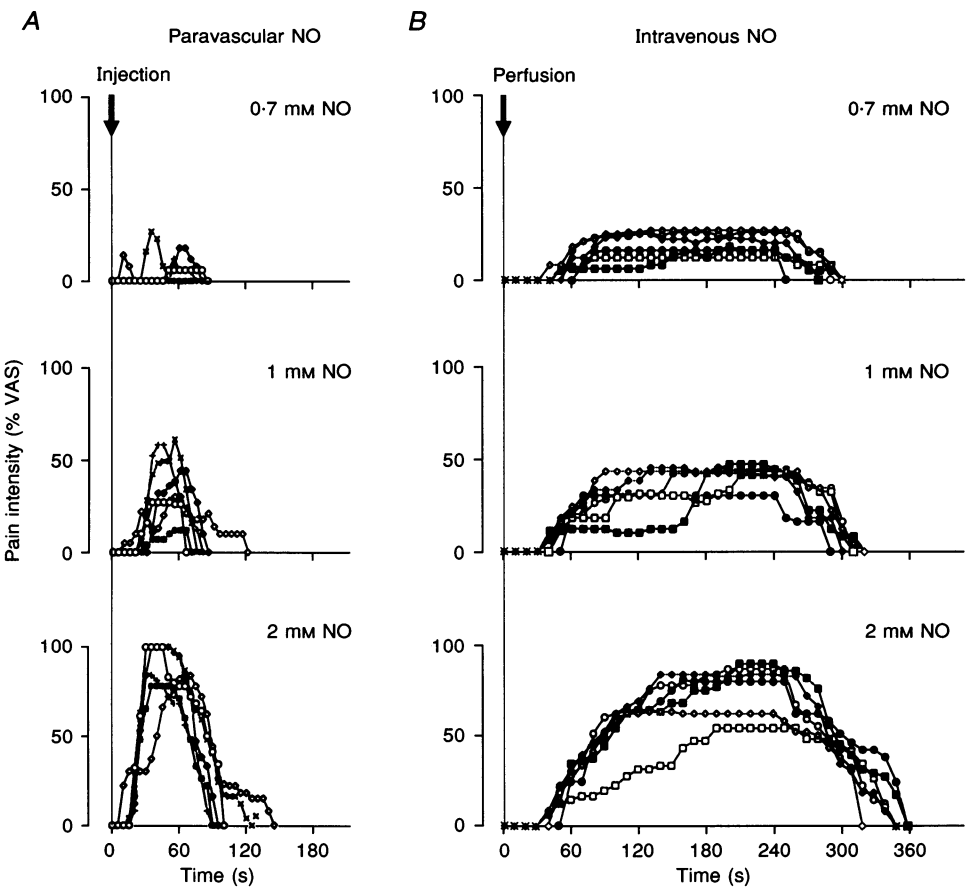

Figure 1. Time course of pain on paravascular injection (*A*) and intravenous perfusion (*B*) of NO solutions in humans

Pain was rated on a visual analog scale (VAS) between 0% VAS (no pain) and 100% VAS (maximally tolerable pain). Shown are the pain intensity–time curves of each of six subjects. Both intensity and duration of pain increased with concentration of NO regardless of its application site. On intravenous perfusion, pain attained a plateau which was maintained until perfusion was stopped.

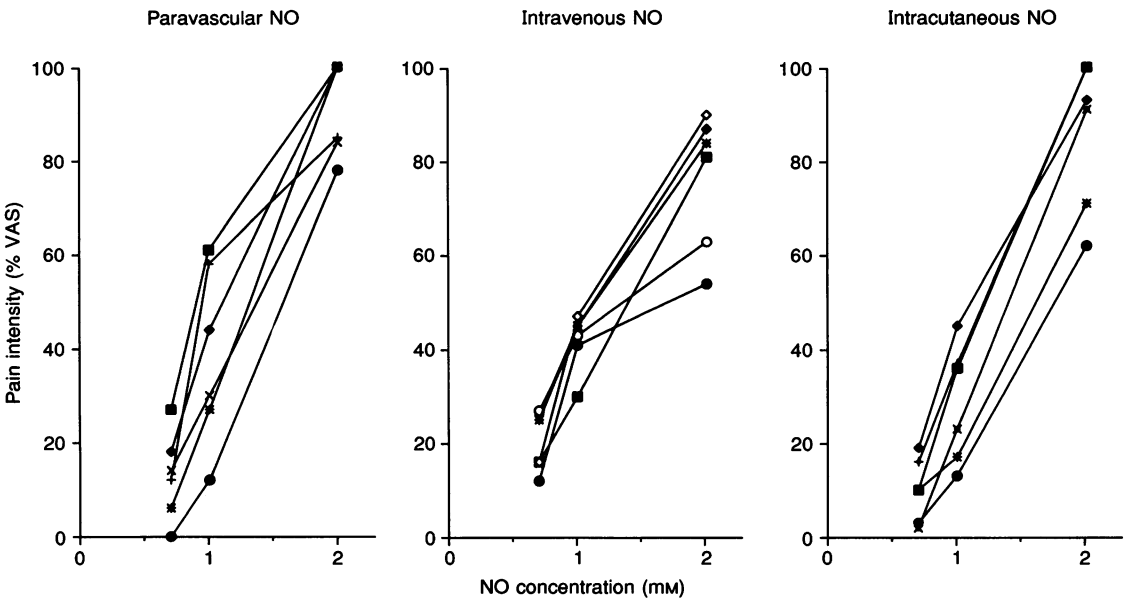

Figure 2. Pain intensity–NO concentration relations for paravascularly, intravenously and intracutaneously applied NO

Data for intracutaneously applied NO is taken from Holthusen & Arndt, 1994. All curves are monotonic, and maximal tolerable pain was usually reached at four to five times the threshold concentration. Note the similarity of the algetic concentration range and the congruency of the curves.

Since pain always occurred with 0·7 mM NO (except in one experiment), but never with 0·4 mM, the threshold concentration must be between these values so that the tolerance maxima were reached at about four to five times the threshold concentration.

Finally, neither oedema nor any other sequelae were observed.

DISCUSSION

In humans, NO evokes pain in a concentration-dependent manner on both paravascular and intravenous application, similar to that evoked on intracutaneous injection. In our experiments, the algetic concentrations were in a similar range and the pain intensity–NO concentration curves were roughly congruent, indicating a similar sensitivity of the nociceptor systems involved.

This conclusion rests on the following premises. First, pain was evoked by authentic NO, i.e. neither by oxidation products of NO, such as $N_xO_y^{n-}$ (Wink, Darbyshire, Nims, Saavedra & Ford, 1993), nor by unphysiological osmolality or hydrogen ion concentrations of the solution. Second, application routes were appropriate to bring NO into contact with nociceptors of both the paravascular tissue and veins. Third, the concentration–effect relations reflect the sensitivity of the different nociceptor populations.

(1) All the solutions were isosmolar and at physiological pH, so that unphysiological osmolality or pH as potential noxious stimuli (Klement & Arndt, 1991) were excluded. In fact, applications of the vehicle solution never evoked pain. Furthermore, NO, which in oxygenated solutions has a very short half-life, was freshly dissolved in a deoxygenated vehicle, where it is stable for weeks. Therefore, the formation of NO oxidation products in the solution is unlikely. To circumvent the problems associated with the short half-life of NO, it would appear that NO precursors or NO donors could have been a better choice than authentic NO. However, NO precursors act analgetically because of the formation of an endogenous neuropeptide (Kawabata, Fukuzumi, Fukushima & Takagi, 1992). In fact, perfusion of isolated hand veins with L-arginine, the NO precursor substance, does not evoke pain in humans (Kindgen-Milles & Arndt, 1993). Also, 3-morpholino-syndonimine and dinatrium-pentacyano-nitrosylferrat, two clinically used NO donors, do not evoke pain (authors' unpublished observations). The failure of the latter substances to evoke pain from veins is probably explained by their limited ability to diffuse through the barrier of endothelium and basement membrane or the possibility that the release of NO is too small in relation to its rapid degradation in oxygenated tissues, so that algetic concentrations of NO are not reached at the nociceptors.

(2) Both routes of application are direct approaches to the respective nociceptors. On paravascular injection, drugs are applied close to nociceptors, as is also evidenced by the short latencies in our experiments. Spreading to cutaneous nociceptors is unlikely, since the basement membrane is an

effective diffusion barrier that prevents paravascularly applied drugs from reaching cutaneous nociceptors (Khasar, Green & Levine, 1993). Finally, backflow of paravascularly applied solution to cutaneous nociceptors is unlikely, since the cannulae were advanced through a small vein so that NO could hardly have reached these receptors via the insertion channel.

During intravenous perfusion, drugs undoubtedly reach venous nociceptors exclusively. Pain is the only sensation evoked by intrasegmental application of various kinds of physicochemical stimuli unless the vein is numbed, regardless of the state of innervation of the surrounding tissue (Arndt & Klement, 1991). Therefore, our application routes were appropriate for NO to reach nociceptors at the intended sites.

(3) We do not know the concentrations of NO at the nociceptors. In general, for a given rate of removal, the concentration of a drug at its site of action will depend on the diffusion gradient and hence on the distance between the site of application and the target. This distance ought to have been relatively short in our experiments. This was certainly the case for the paravascular injection, and for intravenous application, the distance was a few micrometres at most, since the sensory structure involved lies just adjacent to the abluminal side of the endothelium (Flöel & Staubesand, 1978).

At first glance, therefore, it is puzzling that NO concentrations in the millimolar range were needed for evoking pain under these circumstances. This is probably explained by the very high degradation rate of NO in tissues. In the presence of oxygen, NO disappears with a half-life of less than 1 s (Kelm & Schrader, 1990; Ignarro, Fukuto, Griscavage, Rogers & Byrns, 1993), so that degradation far outweighs the diffusion rate. As a consequence, the concentration of NO at the nociceptors themselves should be much lower than the applied concentration; nevertheless, both concentrations will vary in parallel. The delays of action observed in our experiments reflect in all likelihood the interplay between the diffusion of NO from its application site to the site of action and the degradation of NO. Compatible with this view is the fact that both latency and duration of pain depended on the concentration of NO. Thus, from a methodological point of view, we are confident that genuine NO was in contact with both the paravascular and venous nociceptors and that their responsiveness to NO is reflected by the pain intensity–concentration relations.

The possibility that pain in our experiments could have been evoked indirectly by vascular changes or by the formation of oedema is unlikely. First, although nociceptors in veins are mechanosensitive, noxious mechanical dilatation of veins with an at least threefold increase in diameter is needed to evoke pain (Arndt & Klement, 1991). Second, in general, pharmacologically induced venodilatation is not painful. For example, adenosine, a notorious vasodilator, does not evoke pain even if perfused through isolated hand vein segments in millimolar concentrations (Klement & Arndt, 1992). Third, oedemata *per se* are not painful unless associated with inflammation, in which case NO is released by the action of certain mediators (Moncada & Higgs, 1993). Moreover, inhibition of NO synthesis leads to an increase in vascular permeability (Kubes, 1993), so that – if anything – the opposite should have happened in our experiments. Since we observed neither oedemata nor overt distension of the vein segments, the possibility that pain was evoked indirectly by exciting mechanosensitive nociceptors is remote.

The common feature of our observations resides in the painfulness of both paravascularly and intravenously applied NO solutions of similar concentrations, and in the congruency of the pain intensity–concentration relations. This suggests that the nociceptors of both paravascular tissue and veins, as well as those in the skin (Holthusen & Arndt, 1994), are not only responsive to NO, but also share the same NO sensitivity.

In view of the rather low tissue NO concentrations of maximally 10 μM under physiological circumstances (Wink *et al.* 1993), we are hesitant at present to say that NO plays a physiological role as a mediator of pain in peripheral nociception. Nevertheless, some indirect support for this hypothesis is noteworthy. First, inhibitors of NO synthesis reduce the spike traffic in nociceptive spinal neurons when injected into the corresponding receptive fields in animals (Haley, Dickenson & Schachter, 1992; Dickenson, Haley, Schachter & Chapman, 1992). Second, in humans, bradykinin, an endogenous algetic and well-known liberator of NO (Sung *et al.* 1988), evokes pain from veins unless the pathway of NO synthesis is blocked (Kindgen-Milles & Arndt, 1993). Together, these observations are consistent with the hypothesis that NO is a chemical link in nociception. Regardless of this inference, we have shown here that NO evokes pain in humans on both paravascular and intravenous application and that the respective nociceptive systems are equally sensitive to NO.

Archer, S. (1993). Measurement of nitric oxide in biological models. *FASEB Journal* **7**, 349–360.

Arndt, J. O. & Klement, W. (1991). Pain evoked by polymodal stimulation of hand veins in humans. *Journal of Physiology* **440**, 467–478.

Dickenson, A. H., Haley, J. E., Schachter, M. & Chapman, V. (1992). Electrophysiological approaches to the study of bradykinin and nitric oxide in inflammatory pain. *Agents and Actions Supplement* 38/II, 358–365.

Flöel, H. & Staubesand, J. (1978). Neuroendotheliale Kontakte in der Wand grosser Venen bei Ratte und Katzen; elektronenmikroskopische Befunde. *VASA-Journal of Vascular Diseases* **7**, 114–118.

Haley, J. E., Dickenson, A. H. & Schachter, M. (1992). Electrophysiological evidence for a role of nitric oxide in prolonged chemical nociception in the rat. *Neuropharmacology* **31**, 251–258.

Holthusen, H. & Arndt, J. O. (1994). Nitric oxide evokes pain in humans on intracutaneous injection. *Neuroscience Letters* **165**, 71–74.

Ignarro, L. J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released from artery and vein. *Circulation Research* **65**, 1–21.

Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E. & Byrns, R. E. (1993). Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. *Proceedings of the National Academy of Sciences of the USA* **90**, 8103–8107.

Kawabata, A., Fukuzumi, Y., Fukushima, Y. & Takagi, H. (1992). Antinociceptive effect of L-arginine on the carrageenin-induced hyperalgesia of the rat: possible involvement of central opioidergic systems. *European Journal of Pharmacology* **218**, 153–158.

Kelm, M. & Schrader, J. (1990). Control of vascular tone by nitric oxide. *Circulation Research* **66**, 1561–1575.

Khasar, S. G., Green, P. G. & Levine, J. D. (1993). Comparison of intradermal and subcutaneous hyperalgesic effects of inflammatory mediators in the rat. *Neuroscience Letters* **153**, 215–218.

Kindgen-Milles, D. & Arndt, J. O. (1993). Blockade der Stickstoffmonoxid-Synthese, nicht aber der Prostaglandinsynthese hemmt den Bradykinin-induzierten Venenschmerz am Menschen. *Der Anästhesist* **42**, suppl. 1, S315.

Kindgen-Milles, D., Klement, W. & Arndt, J. O. (1994). The nociceptive systems of skin, paravascular tissue, and hand veins of humans and their sensitivity to bradykinin. *Neuroscience Letters* **181**, 39–42.

Klement, W. & Arndt, J. O. (1991). Pain on I.V. injection of some anaesthetic agents is evoked by the unphysiological osmolality or pH of their formulations. *British Journal of Anaesthesia* **66**, 189–195.

Klement, W. & Arndt, J. O. (1992). Adenosine does not evoke pain from venous and paravascular nociceptors in the human. *Cardiovascular Research* **66**, 186–189.

Krauth, J. (1988). Distribution-free statistics. In *Techniques in the Behavioural and Neural Sciences*, vol. 2, ed. Huston, J. P., pp. 129–135. Elsevier, Amsterdam, New York, Oxford.

Kubes, P. (1993). Nitric oxide-induced microvascular permeability alterations: a regulatory role for cGMP. *American Journal of Physiology* **265**, H1909–1915.

Lim, R. K. S., Liu, C. N., Guzman, F. & Braun, C. (1962). Visceral receptors concerned in visceral pain and the pseudo-affective response to intra-arterial injection of bradykinin and other algesic agents. *Journal of Comparative Neurology* **118**, 269–293.

Meller, S. T. & Gebhardt, G. F. (1993). Nitric oxide (NO) and nociceptive processing in the spinal cord. *Pain* **52**, 127–136.

Moncada, S. & Higgs, A. (1993). The L-arginine–nitric oxide pathway. *New England Journal of Medicine* **329**, 2002–2012.

Steranka, L. R., Manning, D. C., DeHaas, C. J., Ferkany, J. W., Borosky, S. A., Connor, J. R., Vavrek, R. J., Stewart, J. M. & Snyder, S. H. (1988). Bradykinin as a pain mediator: Receptors are localized to sensory neurons, and antagonists have analgesic actions. *Proceedings of the National Academy of Sciences of the USA* **85**, 3245–3249.

Sung, C. P., Arleth, A. J., Shikano, K. & Berkowitz, B. A. (1988). Characterization and function of bradykinin receptors in vascular endothelial cells. *Journal of Pharmacology and Experimental Therapy* **247**, 8–13.

Wink, D. A., Darbyshire, J. F., Nims, R. W., Saavedra, J. E. & Ford, P. C. (1993). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: Determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chemical Research in Toxicology* **6**, 23–27.

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