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# THE PROTECTIVE ACTION OF INOSINE ON ISOLATED ARTERIES IN HYPOXIA

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**1** The pressor responses to injected noradrenaline (NA) of isolated perfused femoral or renal arteries of the rabbit were studied.

**2** Vascular smooth muscle is relatively resistant to hypoxia. A combination of hypoxia and dinitrophenol (DNP) respiratory uncoupling was necessary to abolish the pressor response to NA. Loss of the pressor response was assumed to result from decreased capacity of arteries to form adenosine 5'-triphosphate (ATP). Reperfusion of the hypoxic arteries with oxygenated medium resulted in recovery of the pressor response to NA.

**3** Inclusion of inosine (10 mM) in the hypoxic perfusion medium increased significantly the rate and extent of post-hypoxic recovery of the pressor response to NA.

**4** Whereas the presence of inosine in the hypoxic perfusion medium aided post-hypoxic recovery, inosine had no direct action on the pressor dose response to NA. Therefore, the action of inosine was protective as opposed to direct.

**5** The protective action of inosine did not involve potentiation of NA binding to NA-adrenoceptor sites (the equilibrium coefficient, $K_{eq}$ for NA-receptor interaction was unaltered by hypoxia and/or inosine).

**6** The results are discussed in terms of a presumptive mechanism whereby inosine is believed to act by maintaining intracellular adenine nucleotide concentrations in hypoxia.

## Introduction

Ischaemic cellular damage is due to a multiplicity of factors, such as lactic acidosis, ion imbalance and the lack of glycolytic substrates. In ischaemia or hypoxia, the transition from aerobic to anaerobic metabolism results in decreased intracellular adenosine 5'-triphosphate (ATP) concentrations and increased catabolism of adenine nucleotides, i.e. ATP, adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP). The products of catabolism, the purine nucleosides adenosine and inosine and the oxypurine hypoxanthine are released into the bloodstream (Imai, Riley & Berne, 1964; Rubio & Berne, 1975). It has been shown, in certain instances, that the extent of adenine nucleotide catabolism (and consequent purine base efflux) may be correlated with the extent of irreversible tissue damage (Buhl & Jörgensen, 1975). Adequate intracellular adenine nucleotide concentrations are a pre-requisite for metabolic activity. If ischaemia or hypoxia is prolonged, intracellular concentrations of adenine nucleotides and their catabolites are decreased. Thus, metabolic activity and cellular integrity is jeopardised by the lack of ATP.

Following a period of hypoxia, cells are able to resynthesize adenine nucleotides from purine nucleosides. It would seem then, that if intracellular purine nucleoside concentrations could be maintained during ischaemia or hypoxia, then the cells' post-hypoxic capability of resynthesizing adenine nucleotide would be enhanced. In this study we have tried to determine if by raising the extracellular concentration of inosine, tissues could be protected from the irreversible effects of hypoxia. If inosine, following a period of hypoxia, is incorporated into cellular adenine nucleotide, then tissues should display an enhanced capacity for recovery.

## Methods

The apparatus used in this study has been described previously (de la Lande & Rand, 1965; Bloom, McCalden & Rosendorff, 1975). Male Cross lop rabbits (2.5 to 3.0 kg) were anaesthetized with intravenous sodium pentabarbitone (Nembutal, Abbott Laboratories). Lengths (2 to 2.5 cm) of femoral or

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renal arteries were cannulated proximally, removed from the animals and placed on the perfusion apparatus. Arteries were then perfused with Krebs-Ringer bicarbonate (Krebs & Hanseleit, 1932) at pH 7.4 and containing 5 mM glucose (KRB-Glucose) at constant flow (3.5 ml/min), in an organ bath at 37°C. Perfusion pressure was monitored in each experiment with a pressure transducer (Statham P37) connected upstream to the artery via a T-tube. Arterial vasoconstrictor responses to bolus doses of NA injected through a rubber septum immediately proximal to the artery were monitored as changes in perfusion pressure, which gave indirectly an indication of the changes in arterial resistance.

Six experiments were performed in which pressor dose-response curves of isolated arteries to NA (0.2 to 10 μg) were determined before and after a period of hypoxic perfusion. Isolated arteries were perfused with KRB-Glucose gassed with 95% $O_2$ and 5% $CO_2$. After a 30 min period of equilibration, pressor dose-responses to NA were obtained. The changes in arterial calibre following doses of NA were measured as the changes in pressure from the resting basal tone. This provided the control response curve. The arteries were then perfused with KRB-Glucose gassed with 95% $N_2$ and 5% $CO_2$ and containing 100 μM dinitrophenol (DNP). In the absence of direct measurements the combination of hypoxia and respiratory uncoupling was considered necessary to deplete intracellular adenine nucleotide levels. Vascular smooth muscle was found to be relatively resistant to hypoxia, presumably because glycolytic and residual respiratory flux produced ATP at a rate sufficient to meet the immediate energy demands of the tissue. DNP-mediated respiratory uncoupling was necessary in combination with hypoxia to produce zero pressor responses to NA. This was obtained by giving repeated bolus doses of NA (10 μg) until no pressor response was elicited. The arteries were then reperfused with KRB-Glucose gassed with 95% $O_2$ and 5% $CO_2$. After 5 min of reperfusion, pressor responses to 0.2 to 10 μg NA were obtained. In each experiment measurements were again taken from the resting basal tone, which was the same as that of the control curve. The resultant dose-response curves provided an index of post-hypoxic recovery. The arteries were once again perfused with KRB-Glucose gassed with 95% $N_2$ and 5% $CO_2$ and containing 100 μM DNP, but now 10 mM inosine was added to the perfusion fluid. After zero responses to NA were obtained, recovery was initiated by reperfusion with oxygenated KRB-Glucose. After 5 min, pressor dose-response curves to NA were obtained and measured in a similar manner to the previous curves. The response curves obtained provided an index of post-hypoxic recovery after exposure to inosine. On occasions, the order of the experiment was reversed, i.e. recovery from hypoxia in the presence of inosine was examined first and recovery from hypoxia in the absence of inosine second. These experiments were designed to ascertain whether inosine improved recovery from hypoxia.

Five further experiments were then performed to ascertain whether inosine had any effect on the rate of recovery from hypoxia. In these experiments, arteries were again perfused with KRB-Glucose gassed with 95% $O_2$ and 5% $CO_2$ and 10 bolus doses of a submaximal fixed concentration of NA (2 to 5 μg) were given. This provided a range of control responses to NA. The arteries were then perfused with KRB-Glucose gassed with 95% $N_2$ and 5% $O_2$ and containing 100 μM DNP and repeated 10 μg doses of NA were given until zero response was elicited. Arteries were then reperfused with 95% $O_2$ and 5% $CO_2$ and bolus doses of 2 to 5 μg NA were given repeatedly at fixed intervals until it was judged that the responses to NA had reached a plateau. Zero responses were again obtained by repeated 10 μg NA doses to arteries perfused with 95% $N_2$ and 5% $CO_2$ gassed KRB-Glucose containing 100 μM DNP, but now with 10 mM inosine added to the perfusion fluid. The responses to repeated bolus doses of 2 to 5 μg NA were then obtained with 95% $O_2$ and 5% $CO_2$ gassed KRB-Glucose as before. In each experiment measurements were made from the resting basal tone. These experiments provided an index of the rate of recovery of arteries from hypoxia in the presence and absence of inosine.

Finally, a further five experiments were performed to ascertain whether inosine exerted any direct effects on arteries. Pressor-dose responses were obtained to bolus doses of 0.2 to 10 μg NA in a KRB-Glucose solution gassed with 95% $O_2$ and 5% $CO_2$. These pressor dose-responses were then repeated in the same medium in the presence of 10 mM inosine.

## Results

### *The effect of inosine on the magnitude of post-hypoxic recovery*

Six experiments were performed to investigate the effect of inosine on post-hypoxic recovery. In none of the experiments was there any change in basal tone with hypoxia, DNP and/or inosine. It was shown that inosine did exert a protective effect on the arteries; recovery from hypoxia, in the presence of inosine, being significantly greater than in its absence. Figure 1 shows the dose-response curves from a typical experiment. Curve C represents the control dose-responses to NA in oxygenated KRB-Glucose. The dose-response curves performed after hypoxia and DNP-mediated respiratory uncoupling in the presence and

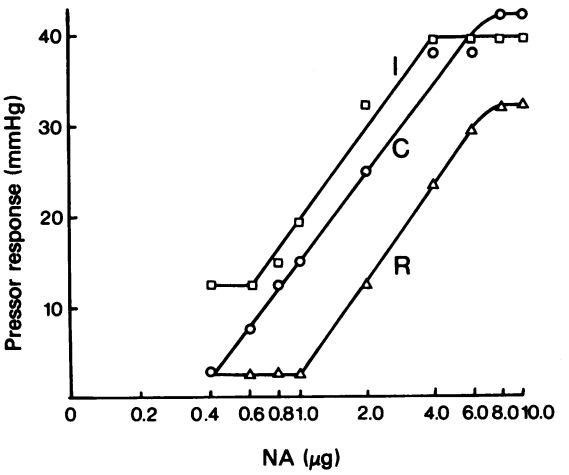


**Figure 1** The pressor response (mmHg) of a typical isolated artery preparation to noradrenaline (NA, 0–10 μg). Pressor dose-response to NA was examined after equilibration in oxygenated KRB-Glucose (○: curve C), after recovery from hypoxia and dinitrophenol (DNP)-mediated respiratory uncoupling (△: curve R) and again after recovery from hypoxia and DNP-mediated respiratory uncoupling in the presence of inosine (10 mM) (□: curve I). Each of the curves represent changes in pressure from the resting basal tone which was the same in each case. For details, refer to text.

absence of inosine are shown in curves I and R respectively.

The results of all six experiments are summarized in Table 1. Here it can be seen that the recovery responses obtained when inosine was included during the hypoxic phase are significantly greater than when it was absent, in five of the six experiments.

The data obtained in each experiment were also plotted as the reciprocal of the pressor response ($^1$/response, $^1$/R) versus the reciprocal of the dose of NA ($^1$/dose, $^1$/D). Straight line relationships in each experiment were obtained, as predicted from Michaelis-Menten kinetics for drug receptor interaction (Goldstein, Aronow & Kaplan, 1968).

$$\frac{1}{R} = \frac{K_{eq}}{R_{max}} \times \frac{1}{D} + \frac{1}{R_{max}}$$

where $R_{max}$ = maximal response and $K_{eq}$ = equilibrium coefficient for the drug-receptor interaction. In each experiment, values of $R_{max}$ and $K_{eq}$ were calculated from the intercepts on the abscissa and ordinate axes, respectively, of the double reciprocal plots.

Changes in $R_{max}$ imply changes in the maximal response of the arteries to NA, whereas changes in $K_{eq}$ imply changes in NA receptor affinity. A graph obtained from a typical experiment is shown in Figure 2. It can be seen that hypoxia had no effect

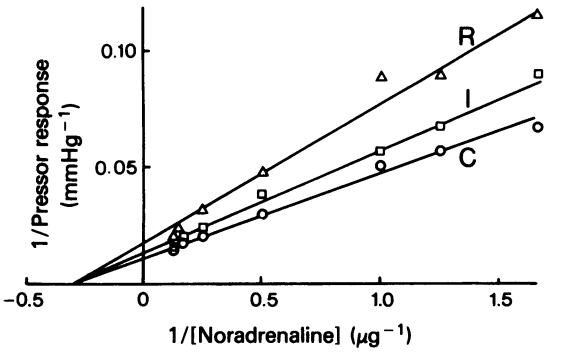


**Figure 2** Double reciprocal plots (1/pressor response, mmHg$^{-1}$ versus 1/NA concentration, μg$^{-1}$) of pressor dose-response curves to noradrenaline (NA) of a typical isolated artery preparation. Dose-response to NA was examined after equilibration in oxygenated KRB-Glucose (○: curve C), after recovery from hypoxia and dinitrophenol (DNP)-mediated respiratory uncoupling (△: curve R) and again after recovery from hypoxia and DNP-mediated respiratory uncoupling in the presence of inosine (10 mM) (□: curve I).

on $K_{eq}$; curves C, I and R all tend to a point on the 1/D axis. However, changes in $R_{max}$ are evident. Recovery from hypoxia results in an apparent decrease in $R_{max}$, this decrease being far less pronounced when inosine was included during the hypoxic phase. These differences in $R_{max}$ for all six experiments are summarized in Table 2. The first column, C > R, shows the statistical probability that the control dose-response curve, C, was greater than the dose-response curve after hypoxia in the absence of inosine, R. All six experiments show that C was greater than R, a necessary finding if the validity of the hypothesis was to be tested. The second column compares control curves, C, with curves obtained after recovery from hypoxia in the presence of inosine, I. The *P* values obtained show that in three experiments, there was no significant difference between the control curves and the recovery curves following hypoxia in the presence of inosine. In the other three experiments, the recovery curves failed to attain control values. The last column compares the recovery curves from hypoxia. The *P* values are given for I > R and show that in five of the six experiments the recovery from hypoxia was greater when inosine was included in the hypoxic perfusate.

*The effect of inosine on the rate of recovery*

A series of five experiments showed that inosine improved significantly the rate of recovery from hypoxia ($P < 0.001$–$0.05$, Student's *t* test on analysis of variance).

The results of a typical experiment are shown in Figure 3. Here it can be seen that following hypoxia,

**Table 1** Recovery response (mmHg) from hypoxia in the presence of inosine minus recovery response (mmHg) in the absence of inosine

| *Experiment number* | *Dose noradrenaline (μg)* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *0.2* | *0.4* | *0.6* | *0.8* | *1.0* | *2.0* | *4.0* | *6.0* | *8.0* | *10.0* | P *values* |
| 1 | +2.5 | +2.5 | 0 | +7.5 | +7.5 | +10.0 | +12.5 | +15.0 | +17.5 | +17.5 | 0.001 |
| 2 | — | +5.0 | +10.0 | +17.5 | +12.5 | +15.0 | +7.5 | +60.0 | +22.5 | 0 | 0.05 |
| 3 | 0 | +0.5 | +2.5 | +3.7 | +6.2 | +8.7 | +10.0 | +10.0 | +15.0 | +10.0 | 0.01 |
| 4 | — | +10.0 | +10.0 | +12.5 | +15.0 | +20.0 | +15.0 | +10.0 | +2.5 | +7.5 | 0.001 |
| 5 | +2.5 | +2.5 | +5.0 | +7.5 | +2.5 | +2.5 | 0 | +7.5 | +2.5 | 0 | 0.01 |
| 6 | 0 | 0 | 0 | +5.0 | 0 | 0 | 0 | 0 | +2.5 | +2.5 | NS |

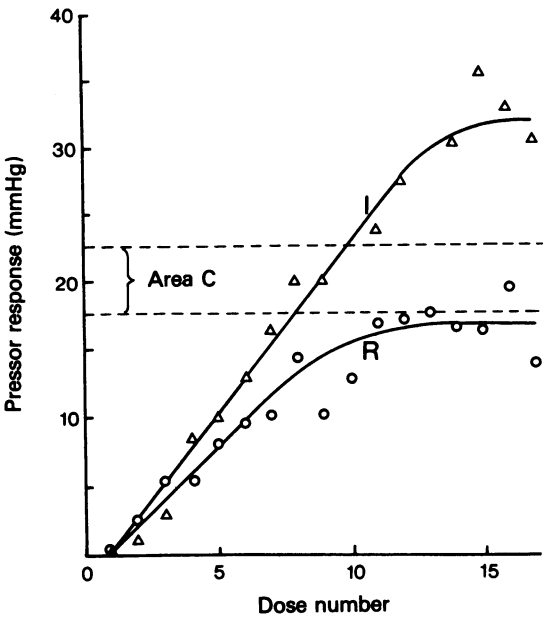


**Figure 3** The time-dependent recovery of the pressor response (mmHg) to a fixed submaximal dose of noradrenaline (NA, in this instance 2 μg) of a typical isolated artery preparation. At the outset of the experiment, 10 bolus doses of 2 μg NA were given to the artery, equilibrated in oxygenated KRB-Glucose, to establish the range of control pressor responses (area C). Recovery of the pressor response to NA (2 μg) from hypoxia and dinitrophenol-mediated respiratory uncoupling in the presence (△: curve I) and absence (○: curve R) of inosine (10 mM) was followed. Successive doses of NA were given at defined time intervals (0.83 min) after recovery from the preceding dose. Thus, dose number is an index of time since hypoxia.

**Table 2** Analysis of differences in data deriving $R_{max}$ values

| | *Statistical probabilities*, P | | |
|---|---|---|---|
| *Experiment* | $C > R$ | $C > I$ | $I > R$ |
| 1 | 0.001 | 0.05 | 0.01 |
| 2 | 0.05 | N.S. | 0.05 |
| 3 | 0.001 | N.S. | 0.001 |
| 4 | 0.001 | N.S. | 0.001 |
| 5 | 0.001 | 0.05 | 0.01 |
| 6 | 0.001 | 0.001 | N.S. |

The statistical significance of the differences in $R_{max}$ between control (C), recovery from hypoxia in the absence of inosine (R) and recovery from hypoxia in the presence of inosine (I). The probabilities (P values) are listed for C being significantly greater than R (C > R), C being significantly greater than I (C > I) and I being significantly greater than R (I > R).

in the presence of inosine, recovery to control levels (area C) was reached (curve I) much faster than when it was absent (curve R). These responses were to the same fixed sub-maximal dose of NA. It can be seen that curve I plateaus at a higher response range than curve R; a result to be expected from the preceding series of experiments. It can also be seen that curve I plateaus above the control response area with the dose of NA used in this experiment. This latter finding was variable in the other experiments. Sometimes curve I attained values higher than control values and sometimes lower. In no instance did curve R ever attain control values.

*The direct effect of inosine*

In five experiments, dose-response curves were obtained in oxygenated KRB-Glucose and these were compared with those obtained with oxygenated KRB-Glucose plus 10 mM inosine. No significant differences were found in the responses to NA in the presence or absence of inosine.

## Discussion

The results show that, in line with the working hypothesis, it was possible to protect vascular smooth muscle from the effects of hypoxia and respiratory uncoupling by exposure of the tissue to inosine during the hypoxic period. Arteries which had been exposed to inosine, recovered substantially faster and to a greater degree than when inosine was absent.

The working hypothesis postulates that inosine acts primarily by maintaining intracellular adenine nucleotide concentrations. The results obtained support this viewpoint, although the evidence is circumstantial. The first presumed reaction in inosine metabolism is phosphorolysis to produce hypoxanthine and ribose l-phosphate. Hypoxanthine is a precursor in adenine nucleotide biosynthesis via the so-called 'salvage pathways', whereas ribose l-phosphate may either be channelled into the hexose monophosphate shunt and glycolysis or into the synthesis of 5-phosphoribosyl l-pyrophosphate (PRPP). PRPP, like hypoxanthine, is a substrate in adenine nucleotide biosynthesis. The channelling of ribose l-phosphate into glycolysis could provide an important anaerobic energy source and this mechanism would appear to be the primary mode of action of inosine on isolated rat lymphocytes (Cole, Stewart & Palmer, 1977; Nordeen & Young, 1977). However, in the artery preparation this mechanism is unlikely to be of major importance. The glucose (5 mM) present in all perfusion media, it is argued, should provide an adequate glycolytic substrate supply. Metabolism of ribose

l-phosphate, therefore, is unlikely to increase glycolytic flux to any significant extent.

The double reciprocal plots show that inosine was able to affect $R_{max}$ without altering $K_{eq}$. These findings suggest that inosine action is not a function of altered NA binding or NA affinity for adrenoceptors (Juhász-Nagy & Aviado, 1977). In addition, as the protective action of inosine in hypoxia was estimated in its absence (i.e. the recovery perfusate contained no inosine), it is unlikely that inosine action could be by direct stimulation of glucose uptake and glycolysis as suggested by Kypson & Hait (1976).

The most likely mechanism of action of inosine is by maintenance of an intracellular pool of adenine nucleotide precursors during hypoxia. The return to control oxygenated perfusion allows a rapid resynthesis of adenine nucleotide pools, with a concomitant improvement in pressor-response to NA. The findings that inosine improves $R_{max}$ with no effect on $K_{eq}$, that inosine improves the rate and magnitude of recovery, that its action is indirect and that it is devoid of any direct effect, all support such a mechanism. The maximal pressor response obtained after recovery from hypoxia was, on occasions, significantly greater than the initial control response (see, for example, Figure 3). This result, which might appear at first glance anomalous, is in fact consistent with the hypothesis if one assumes a variable degree of ischaemic damage to the arteries during surgical removal. Assuming that the intracellular adenine nucleotide concentrations are depleted during surgical removal, inosine would be expected to produce a shift to the left in the dose-response curve (see Figure 1) and, thereby, produce an apparent enhanced pressor response.

The isolated perfused artery was selected purely as a model system. The results have a wider significance. They add to a growing body of evidence which suggests that inosine and related purine nucleosides act *in vitro* and *in vivo* to protect tissues in hypoxia or ischaemia (Fernando, Griffiths, O'Donoghue, Ward, Armstrong, Hendry, Perrett & Wickham, 1976; Kingaby, Lab & Woollard, 1977).

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## References

BLOOM, D.S., MCCALDEN, T.A. & ROSENDORFF, C. (1975). The effects of hypercholesterolaemic plasma on vascular sensitivity to noradrenaline. *Br. J. Pharmac.*, **54**, 421–427.

BUHL, M.R. & JÖRGENSON, S. (1975). Breakdown of 5'-adenine nucleotides in ischaemic renal cortex estimated by oxypurine excretion during perfusion. *Scand. J. clin. Lab. Invest.*, **35**, 211–217.

COLE, A.W.G., STEWART, J.S.W. & PALMER, T.N. (1977). The protective effects of purine nucleosides on the release of intracellular enzymes in hypoxia. *Biochem. Soc. Trans.*, **5**, 1732–1734.

DE LA LANDE, I.S. & RAND, M.J. (1965). A simple isolated nerve-blood vessel preparation. *Aust. J. exp. Biol. Med. Sci.*, **43**, 639–656.

FERNANDO, A.R., GRIFFITHS, J.R., O'DONOGHUE, E.P.N., WARD, J.P., ARMSTRONG, D.M.G., HENDRY, W.F., PERRETT, D. & WICKHAM, J.E.A. (1976). Enhanced preservation of the ischaemic kidney with inosine. *Lancet*, **i**, 555–557.

GOLDSTEIN, A., ARONOW, L. & KAPLAN, S.M. (1968). In *Principles of Drug Action*, p. 70. New York: Hoeber.

IMAI, S., RILEY, A.L. & BERNE, R.M. (1964). Effect of ischaemia on adenine nucleotides in cardiac and skeletal muscle. *Circulation Res.*, **15**, 443–450.

JUHÁSZ-NAGY, A. & AVIADO, D.M. (1977). Inosine as a cardiotonic agent that reverses adrenergic beta blockade. *J. Pharmac. exp. Ther.* **202**, 683–695.

KINGABY, R.O., LAB, M.J. & WOLLARD, K. (1977). Restoration of function in ischaemic myocardium by inosine. *J. Physiol.*, **272**, 102–103P.

KREBS, H. A. & HENSELEIT, J. (1932). Untersuchungen über die Harnstoffbildung im Tierkorper. *Hoppe Seylers Z. Physiol. Chem.*, **210**, 33–66.

KYPSON, J. & HAIT, G. (1976). Effects of uridine and inosine on glucose metabolism in skeletal muscle and activated lipolysis in adipose tissue. *J. Pharmac. exp. Ther.*, **199**, 565–574.

NORDEEN, S.K. & YOUNG, D.A. (1977). Separation of effects of adenosine on energy metabolism from those on cyclic AMP in rat thymic lymphocytes. *J. biol. Chem.* **252**, 5324–5331.

RUBIO, R. & BERNE, R.M. (1975). Regulation of coronary blood flow. *Progr. Cardiovasc. Dis.*, **18**, 107–122.

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