A ação do NAD^+ extracelular sobre a neoglicogênese em fígado de rato

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá, área de concentração em Biologia Celular, para obtenção do grau de Mestre

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Maringá
2005

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Este é um trabalho de equipe, realizado no Laboratório de Metabolismo Hepático da Universidade Estadual de Maringá; os resultados estão descritos e discutidos no artigo:

Martins AG, Constantin J, Bracht F, Kelmer-Bracht AM, Bracht A. The action of extracellular NAD^+ on gluconeogenesis in the perfused rat liver. **Molecular and Cellular Biochemistry** (submetido).

Ficha catalográfica — BCE/UEM

Martins, Adriana Gallego
A ação do NAD^+ extracelular sobre a neoglicogênese em fígado de rato — Maringá: UEM, 2005. 32 p., gráficos.
Diss. (mestrado) — UEM-DBQ, 2005.
Orientadora: Dra. Jorgete Constantin.
1. Fígado em perfusão; 2. nicotinamida adenina dinucleotídeo (NAD^+); 3. ADP-ribose cíclica (ADPRc); 4. neoglicogênese; 5. consumo de oxigênio; 6. eicosanóides; 7. zoneamento.
I. Universidade Estadual de Maringá.

RESUMO

INTRODUÇÃO E OBJETIVOS — Recentemente foi descoberto que o nicotinamida adenina dinucleotídeo (NAD^+) está envolvido na sinalização intracelular. Sistemas enzimáticos bifuncionais (NAD^+ glicohidrolases/ADP-ribosil-ciclases) geram ADP-ribose, nicotinamida livre e ADP-ribose cíclica (ADPRc). Esta última tem sido reconhecida como agente mobilizador de Ca^{2+} em vários tipos de células. As NAD^+ glicohidrolases/ADP-ribosil ciclases, em sua maior parte glicoproteínas transmembrana, foram encontradas em muitos organismos e células, incluindo células do fígado (células de Kupffer).

No fígado de rato em perfusão o NAD^+ causa hipertensão portal e arterial, produz inibição inicial do consumo de oxigênio seguida de estimulação e aumenta a glicogenólise. Estes efeitos são dependentes de Ca^{2+} e resultam de uma interação entre células parenquimais e não-parenquimais via produção de eicosanóides. Estas ações não estão distribuídas uniformemente ao longo do ácino hepático. No caso do consumo de oxigênio, por exemplo, o sinal gerador da inibição está localizado pré-sinusoidalmente, enquanto que o sinal responsável pelo estímulo parece estar distribuído uniformemente ao longo do ácino hepático.

Animais alimentados foram utilizados nos estudos publicados até agora acerca dos efeitos do NAD^+ extracelular sobre o metabolismo hepático. No estado alimentado, a liberação de glicose resulta, basicamente, da glicogenólise, enquanto que no estado de jejum aquela atividade passa a ser devida à neoglicogênese. Além disto, no fígado de ratos em jejum, o metabolismo energético depende em maior grau da oxidação de ácidos graxos. É neste contexto que surge a questão acerca da ação do NAD^+ extracelular sobre a neoglicogênese e o consumo de oxigênio no fígado de ratos em jejum. Em princípio pelo menos, como a neoglicogênese depende de energia, uma inibição do consumo de oxigênio deveria ser acompanhada de inibição da neoglicogênese, assim como um estímulo do consumo de oxigênio deveria resultar em estímulo da neoglicogênese. Neste trabalho, esta questão foi investigada no fígado em perfusão (modos anterógrado e retrógrado), utilizando lactato como precursor gliconeogênico, perfusão livre de cálcio e diversos inibidores da síntese de eicosanóides.

MÉTODOS — Ratos machos Wistar, pesando entre 190 e 220 g, alimentados *ad libitum* com ração padronizada, foram utilizados. O fígado foi perfundido monovascularmente no modo não-recirculante. O líquido de perfusão foi o tampão Krebs/Henseleit-bicarbonato (pH 7,4), saturado com uma mistura de O_2 e CO_2 (95:5) através de um oxigenador de membrana e aquecido simultaneamente a 37°C. Isolamento e canulação do fígado foram feitos sob anestesia de tiopental sódico (50 mg/kg). Dois modos de perfusão foram utilizados: a) anterógrado (veia porta → veia hepática); b) retrógrado (veia hepática → veia porta). A concentração de glicose no perfusado efluente foi medida enzimaticamente com a glicose oxidase. O consumo de oxigênio foi monitorado continuamente através de polarografia. Perfusão livre de cálcio foi feita após depletar os estoques celulares de Ca^{2+}.

RESULTADOS — 1) A infusão de NAD^+ na veia porta (perfusão anterógrada) causou inibição transitória do consumo de oxigênio e da neoglicogênese. A extensão da diminuição foi dependente de concentração na faixa entre 25 e 100 μM. Após a recuperação da inibição, houve estímulo do consumo de oxi-

gênio e da neoglicogênese; este fenômeno foi mais pronunciado na concentração de 50 μM.

2) As variações da neoglicogênese apresentaram correlação parabólica com as variações do consumo de oxigênio durante a infusão de NAD^+ (coeficiente de correlação = 0,91).

3) Após inibição da respiração e da neoglicogênese por rotenona + antimicina A o NAD^+ não teve mais efeito sobre estes parâmetros; na presença de 2,4-dinitrofenol, que suprime a neoglicogênese e estimula o consumo de oxigênio, apenas o efeito inibidor do NAD^+ sobre a respiração ainda se fez presente.

4) A infusão de NAD^+ na veia hepática (perfusão retrógrada) causou estímulos transitórios do consumo de oxigênio e da neoglicogênese sem ter havido uma fase de inibição. Os incrementos máximos do consumo de oxigênio e da neoglicogênese foram iguais a 0,40±0,08 e 0,21±0,03 μmol min^{-1} g^{-1}, respectivamente.

5) Durante a perfusão livre de Ca^{2+} a ação do NAD^+ 100 μM ficou reduzida a um pequeno estímulo transitório do consumo de oxigênio e da neoglicogênese.

6) Na presença de indometacina 30 μM (inibidor da ciclo-oxigenase) as ações do NAD^+ 50 μM sobre o consumo de oxigênio e a neglicogênese foram praticamente abolidas.

7) Na presenca de brometo de bromofenacila 100 μM (inibidor da fosfolipase A_2) as ações inibidoras do NAD^+ 50 μM sobre a neoglicogênese e o consumo de oxigênio foram abolidas, permanecendo apenas pequenas tendências estimuladoras.

8) Na presenca do ácido nordihidroguaiarético 25 μM (inibidor da síntese de leucotrienos) as ações inibidoras do NAD^+ 50 μM sobre o consumo de oxigênio e sobre a neoglicogênese foram substancialmente diminuídas. Além disto, os estímulos do consumo de oxigênio e da neoglicogênese após a recuperação da inibição foram abolidos.

CONCLUSÕES E DISCUSSÃO — Os resultados demonstram que o NAD^+ age também no estado de jejum, atuando sobre o consumo de oxigênio e sobre a neoglicogênese. O mecanismo básico parece ser o mesmo encontrado no estado alimentado. Também no estado de jejum os efeitos são dependentes de Ca^{2+} e parecem ser mediados por eicosanóides provenientes de células não-parenquimais, possivelmente células de Kupffer. Isto é sugerido pela ação supressora ou modificadora dos inibidores da síntese de eicosanóides.

As alterações do consumo de oxigênio causadas pelo NAD^+ refletem alterações no fluxo de elétrons na cadeia respiratória. Isto é indicado pela observação de que não houve mais inibição do consumo de oxigênio quando a cadeia respiratória foi bloqueada por rotenona + antimicina A.

Assim como ocorre no estado alimentado, na condição de jejum o NAD^+ também pode exercer dois efeitos antagônicos, inibição e estímulo. O fato de a inibição não ter se manifestado significativamente em perfusão retrógrada indica que o seu sinal gerador deve situar-se na região pré-sinusoidal.

Provavelmente os efeitos primários do NAD^+ são a inibição e a ativação do consumo de oxigênio. Isto é indicado, principalmente, pela observação de que a inibição do consumo de oxigênio pode ocorrer mesmo em condições nas quais não há neoglicogênese.

ABSTRACT

INTRODUCTION AND AIMS — It is now known that NAD^+ is involved in intracellular signalling by virtue of enzyme-catalyzed transformations. Bi-functional enzymatic systems (NAD^+ glycohydrolases/ADP-ribosylcyclases) generate ADP-ribose, free nicotinamide and cyclic ADP-ribose (cADPR). The latter has been recognized as a Ca^{2+} mobilizing agent in several cell types. The NAD^+ glycohydrolases/ADP-ribosylcyclases, mostly transmembrane glycoproteins, were found in many organisms and cells, including liver cells (especially in Kupffer cells).

In the perfused rat liver NAD^+ increases both portal and arterial resistance, causes an initial inhibition of oxygen consumption followed by stimulation and increases glycogenolysis. These effects are Ca^{2+}-dependent and they seem to result from an interaction between parenchymal and non-parenchymal cells via eicosanoid production. Furthermore, these actions are not uniformly distributed over the liver parenchyma. In the case of oxygen consumption, for example, the signal generating inhibition is located pre-sinusoidally whereas the signal responsible for oxygen uptake stimulation seems to be uniformly distributed over the liver parenchyma.

A 24 hours fasting period induces substantial changes in the hepatic metabolism of rats. In the fed state, glucose release is, basically, consequence of glycogenolysis, whereas in the fasted state this activity is mainly due to gluconeogenesis. Besides this, in the virtual absence of glycogen, the dependence of hepatic energy metabolism from fatty acid oxidation increases. It is within this context that the question about the action of extracellular NAD^+ on gluconeogenesis and oxygen uptake in the liver of fasted rats arises. In principle at least, since gluconeogenesis is energy-dependent, an inhibition of oxygen uptake should be paralleled by an inhibition of gluconeogenesis in the same way as a stimulation of oxygen consumption should produce stimulation of gluconeogenesis. In the present work this question was investigated in the monovascularly perfused rat liver (antegrade and retrograde modes of perfusion) and using lactate as a gluconeogenic precursor, calcium-free perfusion and several inhibitors of eicosanoid synthesis.

METHODS — Male Wistar rats, weighing between 190 and 220 g, *ad libitum* fed with a standard chow diet, were used. The liver was perfused monovascularly in the non-recirculating mode. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of O_2 and CO_2 (95:5) by means of a membrane oxygenator and simultaneously heated to 37°C. Isolation and cannulation of the liver were done under sodium thiopental anesthesia (50 mg/kg). Two perfusion modes were employed: a) antegrade (portal vein $\rightarrow$ hepatic vein); b) retrograde (hepatic vein $\rightarrow$ portal vein). Glucose in the effluent perfusate was assayed by means of an enzymatic procedure. Oxygen uptake was measured continuously by means of polarography. Calcium-free perfusion was done after depleting the cellular Ca^{2+} stores by means of successive phenylephrine pulses.

RESULTS — 1) NAD^+ infusion into the portal vein (antegrade perfusion) produced transient inhibition of both oxygen uptake and gluconeogenesis. The extent of the diminution was concentration dependent in the range

between 25 and 100 μM. After the recovery from inhibition, stimulation of both oxygen uptake and gluconeogenesis occurred; this phenomenon was more pronounced at a concentration of 50 μM.

2) The changes in gluconeogenesis presented parabolic correlation with the changes in oxygen uptake during NAD^+ infusion (correlation coefficient = 0.91).

3) After inhibition of respiration and gluconeogenesis by rotenone + antimycin A, NAD^+ was no longer effective on these parameters; in the presence of 2,4-dinitrophenol, which supresses gluconeogenesis and stimulates oxygen uptake, inhibition of respiration was the only effect of NAD^+.

4) NAD^+ infusion into the hepatic vein (retrograde perfusion) produced transient stimulations of both oxygen uptake and gluconeogenesis without inhibition. Maximal stimulations of oxygen uptake and gluconeogenesis were equal to 0.40±0.08 and 0.21±0.03 μmol min^{-1} g^{-1}, respectively.

5) During Ca^{2+}-free perfusion the action of 100 μM NAD^+ was restricted to small transient stimulations of both oxygen uptake and gluconeogenesis.

6) In the presence of 30 μM indomethacin (cyclooxygenase inhibitor) the actions of 50 μM NAD^+ on oxygen uptake and gluconeogenesis were practically abolished.

7) In the presence of 100 μM bromophenacyl bromide (phospholipase A_2 inhibitor) the inhibitory actions of 50 μM NAD^+ were abolished, remaining only small stimulatory tendencies.

8) In the presence of 25 μM nordihydroguaiaretic acid (leukotriene synthesis inhibitor) the inhibitory actions of 50 μM NAD^+ on oxygen uptake and gluconeogenesis were both substantially diminished. Furthermore, stimulation of gluconeogenesis and oxygen uptake by NAD^+ after recovery from inhibition was abolished.

Conclusions and Discussion — The results demonstrate that NAD^+ also acts in the fasted state, affecting oxygen consumption and glucose synthesis. The basic mechanism of these actions seem to be the same as that one proposed for the fed state. Also in the fasted state the effects are Ca^{2+}-dependent and seem to be mediated by eicosanoids synthesized in non-parenchymal cells, possibly Kupffer cells. This is strongly suggested by the supressor or modifying actions of inhibitors of eicosanoid synthesis.

The alterations in oxygen consumption caused by NAD^+ reflect alterations in the electron flow along the respiratory chain rather than an action on mixed function oxidation. This is indicated by the observation that there was no longer inhibition of oxygen consumption when the respiratory chain was blocked by rotenone + antimycin A.

As it occurs in the fed state, in the fasted condition extracellular NAD^+ is able to exert two antagonic effects, inhibition and stimulation. The fact that inhibition did not manifest significantly in retrograde perfusion indicates that its generating signal is likely to be located in pre-sinusoidal regions.

The primary effects of NAD^+ are probably inhibition and stimulation of oxygen uptake. This is indicated, mainly, by the observation that inhibition of oxygen uptake by NAD^+ can occur even under conditions where no gluconeogenesis occurs.

The action of extracellular NAD^+ on gluconeogenesis in the perfused rat liver

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Running title: Extracellular NAD^+ and hepatic gluconeogenesis.

Abstract

In the rat liver NAD^+ infusion produces increases in portal perfusion pressure and glycogenolysis and transient inhibition of oxygen consumption. The aim of the present work was to investigate the possible action of this agent on gluconeogenesis using lactate as a gluconeogenic precursor. Hemoglobin-free rat liver perfusion in antegrade and retrograde modes was used with enzymatic determination of glucose production and polarographic assay of oxygen uptake. NAD^+ infusion into the portal vein (antegrade perfusion) produced a concentration-dependent (25-100 μM) transient inhibition of oxygen uptake and gluconeogenesis. For both parameters inhibition was followed by stimulation. NAD^+ infusion into the hepatic vein (retrograde perfusion) produced only transient stimulations. During Ca^{2+}-free perfusion the action of NAD^+ was restricted to small transient stimulations. Inhibitors of eicosanoid synthesis with different specificities (indomethacin, nordihydroguaiaretic acid, bromophenacyl bromide) either inhibited or changed the action of NAD^+. The action of NAD^+ on gluconeogenesis is probably mediated by eicosanoids synthesized in non-parenchymal cells. As in the fed state, in the fasted condition extracellular NAD^+ is also able to exert two opposite effects, inhibition and stimulation. Since inhibition did not manifest significantly in retrograde perfusion it is likely that the generating signal is located in pre-sinusoidal regions.

Keywords: extracellular NAD^+; liver; gluconeogenesis; oxygen uptake; eicosanoids.

Introduction

The biological activity of NAD^+ goes beyond its role as a carrier of reducing equivalents in oxidation-reduction reactions. It is now known that NAD^+ is also involved in intracellular signalling by virtue of enzyme-catalyzed transformations [1-3]. Bifunctional enzymatic systems (NAD^+ glycohydrolases/ADP-ribosyl-cyclases) generate ADP-ribose, free nicotinamide and cyclic ADP-ribose (cADPR). The latter has been recognized as a Ca^{2+} mobilizing agent in several cell types [3-5]. The NAD^+ glycohydrolases/ADP-ribosyl-cyclases, mostly transmembrane glycoproteins, were found in many organisms and cells, including liver cells (especially in Kupffer cells) [3-5]. This means also that extracellular NAD^+ is required for their activity, a property which seems to be complementary to the proposed role of connexin 43 (Cx43) in causing localized NAD^+ extrusions and, thus, localized rises in extracellular NAD^+ [6-8]. Connexin 43 is expressed by non-parenchymal liver cells, including Kupffer cells, stellate cells and endothelial cells [9-11]. Furthermore, it is also believed that bursts of high local extracellular NAD^+ levels may occur in consequence of various pathophysiological conditions such as DNA-damage, oxidative stress and cell lysis in tissue injury and inflammation [12].

It has been shown that the perfused rat liver responds very intensely to an infusion of NAD^+ [13]. Increases in portal and arterial pressure, initial inhibition of oxygen consumption followed by stimulation and increase in glycogenolysis were some of the observed actions. These effects are Ca^{2+}-dependent and they seem to result from an interaction between parenchymal and non-parenchymal cells via eicosanoid production. Furthermore, these actions are not uniformly distributed over the liver parenchyma [14]. In the case of oxygen consumption, for example, the signal responsible for inhibition is generated pre-sinusoidally whereas the signal responsible for stimulation seems to be uniformly distributed over the liver parenchyma.

Livers from fed rats were used in both studies published up to now about the actions of extracellular NAD^+ on hepatic metabolism [13,14]. A 24-hours fast, however, induces substantial changes in the hepatic metabolism of rats. In the fed state, glucose release is, basically, consequence of glycogenolysis, whereas in the fasted state this activity is mainly due to gluconeogenesis [15,16]. Besides this, in the virtual absence of glycogen the dependence of hepatic energy metabolism from fatty acid oxidation increases. It is within this context that the question about the action of extracellular NAD^+ on oxygen uptake and gluconeogenesis in the liver of

fasted rats can be raised. In principle at least, since gluconeogenesis is energy-dependent, an inhibition of oxygen uptake should be paralleled by an inhibition of gluconeogenesis in the same way as a stimulation of oxygen consumption can produce stimulation of gluconeogenesis. In the present work this question was investigated in the monovascularly perfused rat liver in the antegrade and retrograde modes of perfusion and using lactate as a gluconeogenic precursor, calcium-free perfusion and several inhibitors of eicosanoid synthesis.

Materials and methods

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. NAD^+ and all enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St Louis, USA). All standard chemicals were from the best available grade (98-99.8 % purity).

Liver perfusion

Male albino rats (Wistar), weighing 190-220 g, were fed *ad libitum* with a standard laboratory diet (Nuvilab®). For the surgical procedure, the rats were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg). Hemoglobin-free, non-recirculating perfusion was done [17]. After cannulation of the portal and cava veins the liver was positioned in a plexiglass chamber. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted between 30 and 35 ml min^{-1}, depending on the liver weight. In most experiments antegrade perfusion (portal vein → hepatic vein) was performed. In some selected experiments, however, retrograde perfusion (hepatic vein → portal vein) was done. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C. The composition of the Krebs/Henseleit-bicarbonate buffer is the following: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM Na_2SO_4, 1.18 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and 2.5 mM $CaCl_2$.

For performing Ca^{2+}-free perfusion, the intracellular Ca^{2+} pools were exhausted. The following procedure was adopted. Livers were pre-perfused with Ca^{2+}-free Krebs/Henseleit-bicarbonate buffer containing 0.1 mM ethylene diamine tetraacetic acid (EDTA). In order to ensure maximal depletion of the intracellular Ca^{2+} pools, phenylephrine (2 μM) was infused repeatedly (4 times) during short periods of 2 minutes, with intervals of 5 minutes. According to previous reports [18], this procedure depletes the intracellular Ca^{2+}-pools which are normally mobilized when Ca^{2+}-agonists are infused.

Analytical

The oxygen concentration in the effluent perfusate was monitored continuously, employing a teflon-shielded platinum electrode [19]. Samples of the effluent

perfusion fluid were collected according to the experimental protocol and analyzed for their glucose content [20].

Treatment of data

The metabolic rates were expressed as µmol per minute per gram liver wet weight (µmol min^{-1} g^{-1}). Statistical analysis of the data was done by means of the Statistica™ program (Statsoft®, 1998). Monovariate and multivariate variance analyses were done for comparing more than two means. The results are mentioned in the figures as the *p* values.

Results

Figure 1 shows the results of experiments in which the action of NAD^+ on gluconeogenesis and oxygen uptake were measured in perfused livers from 24-hours fasted rats. The perfusion direction was portal vein → hepatic vein (antegrade perfusion). Sampling of the effluent perfusate was initiated after oxygen uptake stabilization (zero perfusion time). Initial glucose release was small, but it increased rapidly after the onset of lactate infusion at 12 minutes. This increase, due to gluconeogenesis, tended to stabilize at 30 minutes perfusion time. Initial oxygen uptake was relatively high due to the oxidation of endogenous fatty acids [17]. It increased further when lactate was infused and also tended to stabilize at 30 minutes perfusion time. NAD^+ infusion into the portal vein produced complex effects on both oxygen uptake and gluconeogenesis. For all NAD^+ concentrations, 25, 50 and 100 μM, initial inhibitions of oxygen uptake and gluconeogenesis occurred. For oxygen uptake this inhibition extended to levels well under the basal rates (i.e., before lactate infusion). The initial inhibition was followed by a rapid recovery which was gradually transformed into stimulation. The latter was also transitory. The peak times for both inhibition and stimulation increased with the NAD^+ concentration, but this change was approximately synchronic for both parameters, gluconeogenesis and oxygen uptake. Table 1 illustrates a quantitative analysis of the peak decreases and increases in gluconeogenesis and oxygen uptake. The peak inhibition increased with the NAD^+ concentration in the range between 25 and 100 μM. The maximal increases of both oxygen uptake and gluconeogenesis, however, were highest at 50 μM NAD^+. In mol per mol terms the peak decreases of oxygen uptake exceeded those of gluconeogenesis by 96%, as can be deduced from the $\Delta(O_2$ uptake$)/\Delta$(gluconeogenesis) ratios. This proportion was approximately the same for all NAD^+ concentrations. On the other hand, the peak increases in oxygen uptake were only approximately 21% above the peak increases in gluconeogenesis for all NAD^+ concentrations.

The similarities in the time courses of the actions of the various NAD^+ concentrations on gluconeogenesis and oxygen uptake revealed by Figure 1 suggest that some kind of correlation should exist between the changes in both variables. The relation between the changes in gluconeogenesis and oxygen uptake was different during inhibition and stimulation as revealed by Table 1. The way by which these relations have changed suggests parabolic rather than linear correlation. An analysis of this possibility is presented in Figure 2 in which the changes in

gluconeogenesis (Y) were represented against the changes in oxygen uptake during NAD^+ infusion at the concentrations of 25, 50 and 100 μM. Negative values correspond to inhibition and positive values to stimulation. It is to be remarked that when X is zero, the parabolic correlation line predicts Y = –0.020±0.012, i.e., no significant change in gluconeogenesis can be expected as long as no change in oxygen uptake takes place.

Figure 3 shows the action of NAD^+ in the presence of rotenone plus antimycin A. With these compounds it is possible to inhibit the electron flow along the mitochondrial respiratory chain without significant effects on mixed function oxidation [21]. Inhibition of oxygen uptake by rotenone plus antimycin A was paralleled by a complete supression of glucose release. The subsequent infusion of NAD^+ did not produce any changes in both oxygen uptake and glucose production.

Figure 4 shows experiments with the 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation [22]. As expected, 2,4-dinitrophenol stimulated oxygen uptake and inhibited gluconeogenesis in consequence of the diminished ATP availability [22]. A small shoulder in the glucose production curve after the strong and rapid initial diminution could be reflecting glucose release from residual glycogen. Stimulation of oxygen uptake was very intense and the oxygen concentration in the outflowing perfusate was reduced to less than 5% of the inflowing concentration (0.86 mM). The introduction of 100 μM NAD^+ produced a transient inhibition. The peak diminution, 0.65±0.16 μmol min^{-1} g^{-1}, was less pronounced than that one found in the absence of the uncoupler (see Table 1). Glucose production, on the other hand, remained minimal during the whole period of NAD^+ infusion.

In livers from fed rats it has been shown that NAD^+ does not produce oxygen uptake inhibition in retrograde perfusion, i.e., when NAD^+ is infused into the hepatic vein [14]. In order to find out if this phenomenon also occurs in the fasted state and how it eventually influences gluconeogenesis, retrograde perfusion experiments were done with 100 μM NAD^+ infusion into the hepatic vein. The mean results of these experiments are shown in Figure 5 and they reveal that inhibition was absent from both gluconeogenesis and oxygen uptake. Stimulation, however, was found. The maximal increases in gluconeogenesis and oxygen uptake were, respectively, 0.21±0.04 and 0.40±0.08 μmol min^{-1} g^{-1}. These changes yield a $\Delta(O_2$ uptake$)/\Delta$(gluconeogenesis) ratio of 1.90. Compared with antegrade perfusion, thus, maximal stimulation tended to be more pronounced (see values for 100 μM NAD^+ in Table 1) and occurred earlier in time (compare time courses of the 100 μM NAD^+ curves in Figure 1).

Figure 6 shows the results of experiments that were done in order to test the Ca^{2+}-dependence of the action of NAD^+. Confirming previous reports [23] depletion of the cellular Ca^{2+} stores and absence of this cation in the perfusion fluid did not significantly affect oxygen uptake and gluconeogenesis from lactate in the absence of hormones. The action of NAD^+, however, was clearly changed by Ca^{2+}-free perfusion in that the inhibitory effects of NAD^+ on both gluconeogenesis and oxygen uptake were abolished. At nearly the same times at which the inhibitions were found in the control condition, small stimulations took place. The maximal increments in gluconeogenesis and oxygen uptake were 0.17±0.05 and 0.18±0.04 μmol min^{-1} g^{-1}, respectively. These maximal increments are similar to those ones found with 100 μM NAD^+ in the presence of Ca^{2+} (see Table 1), but they occurred at earlier times.

Figures 7, 8 and 9 illustrate the actions of three inhibitors of eicosanoid synthesis on the actions of extracellular NAD^+. Indomethacin (30 μM), which is a cyclo-oxygenase inhibitor [24], did not significantly affect oxygen uptake and gluconeogenesis although there was some tendency toward lower rates of gluconeogenesis (Figure 7). The actions of 50 μM NAD^+ on gluconeogenesis and oxygen uptake were considerably reduced by indomethacin. This is true for both phenomena, the initial inhibition and the subsequent stimulation. Bromophenacyl bromide (100 μM), which is a phospholipase A_2 inhibitor [25], did not significantly affect oxygen uptake but it had a small inhibitory effect on gluconeogenesis (Figure 8). In the presence of this compound the inhibitory actions of 50 μM NAD^+ were abolished, remaining only small stimulatory tendencies. Nordihydroguaiaretic acid (25 μM), a leukotriene synthesis inhibitor [26], did not significantly affect oxygen uptake but it had a small inhibitory effect on gluconeogenesis (Figure 9). In the presence of this compound the inhibitory actions of 50 μM NAD^+ on oxygen uptake and gluconeogenesis were both substantially diminished. Furthermore, stimulation of gluconeogenesis and oxygen uptake by NAD^+ after recovery from inhibition was abolished.

Discussion

The results of the present study demonstrate that NAD^+, or one of its metabolites [1-3], also acts in the fasted state, affecting oxygen consumption and glucose synthesis in the rat liver. Similar to some actions in the fed state, extracellular NAD^+ exerts two opposite effects in livers from fasted rats, inhibition and stimulation, each one predominating during different times after starting infusion [13,14]. Other similarities to the effects that were reported for the fed state are their Ca^{2+}-dependence, their sensitivity to various inhibitors of eicosanoid synthesis [13] and their apparent unequal distribution along the hepatic acinus [14].

The effects of NAD^+ on oxygen uptake are most probably due to changes in electron flow along the respiratory chain rather than changes in mixed function oxidation. This is indicated by the observation that NAD^+ was no longer able to affect oxygen uptake when the respiratory chain was blocked by rotenone + antimycin A [21]. It looks likely that the changes in oxygen uptake induced by NAD^+ are primary events which exert a great and possibly decisive influence on the rates of gluconeogenesis. The following observations support this interpretation: a) oxygen uptake inhibition and stimulation also occur in substrate-free perfused livers from fed rats whose gluconeogenic activity is minimal [13,14]; b) inhibition of oxygen uptake also took place in the presence of 2,4-dinitrophenol, another condition where gluconeogenesis was totally absent; c) stimulation of both oxygen uptake and gluconeogenesis was the only effect of extracellular NAD^+ in retrograde perfusion; d) the changes in gluconeogenesis and oxygen uptake over the time after initiation of NAD^+ infusion presented a good correlation; e) Ca^{2+}-free perfusion or perfusion in the presence of inhibitors of eicosanoid synthesis produced similar time responses for both oxygen uptake and gluconeogenesis. Taken together all these observations suggest that oxygen uptake inhibition or stimulation are the primary events which determine the changes in gluconeogenesis. Actually it is known that inhibition of respiration always results in gluconeogenesis inhibition [27-29]. The situation is less straightforward for stimulation of oxygen uptake, but it has been shown that in hepatocytes stimulation of the respiratory chain is a key event in the hormonal stimulation of gluconeogenesis [30-32]. Since hepatic respiration depends largely on fatty acid oxidation, an increase in the latter will also provide more acetyl-CoA for the stimulation of pyruvate carboxylase which catalizes a key-step in gluconeogenesis from lactate [32]. Even so, more specific

complementary effects on gluconeogenesis cannot be completely excluded especially in regard to stimulation. In this respect it should be remarked that the correlation between Δgluconeogenesis and ΔO_2 uptake shows no single proportionality over the whole range which goes from maximal inhibition to maximal stimulation. Actually the peak ΔO_2 uptake/Δgluconeogenesis ratios for inhibition and stimulation are clearly different in antegrade perfusion and the parabolic correlation between Δgluconeogenesis and ΔO_2 uptake reflects a gradual transition between these two different relationships which may represent two different metabolic conditions. A Ca^{2+}-dependent increase, as it exists for several agonists [32,33], seems unlikely. Although extracellular NAD^+ induces Ca^{2+} movements and its inhibitory effects were almost abolished when the Ca^{2+} stores were depleted, the stimulatory effects on both oxygen uptake and gluconeogenesis were still present, even though their peak values were shifted to earlier times. This suggests that the stimulatory action of NAD^+ is at least partially independent of Ca^{2+}.

The sensitivity of the action of extracellular NAD^+ to inhibitors of eicosanoid synthesis in the liver from fasted rats was similar to the sensitivity found in livers from fed rats. There is considerable information available about the stimulatory action of eicosanoids on glycogenolysis and glycolysis, which are the typical parameters generally measured in livers of fed rats [34-36]. These observations, in addition to the sensitivity of the same parameters to NAD^+, allowed to conclude that the action of the latter is, partly at least, mediated by eicosanoids released from non-parenchymal cells [14]. There are also reports indicating that eicosanoids are able to affect gluconeogenesis or at least oxygen consumption in several ways. An early report claims that prostaglandin E1 (PGE1) is able to inhibit the incorporation of trace doses of [2-^{14}C]pyruvate into glucose in perfused livers of rats fasted for 48 hours [37]. Similarly, an unspecified prostaglandin E has been reported to increase glucose synthesis from α-ketoglutarate in rat renal cortical tubules [38]. This effect was no longer observable when Ca^{2+} was not added to the incubation medium. Prostaglandin F2α stimulates gluconeogenesis from lactate plus pyruvate in perfused rat livers [39]. In these investigations with livers or renal cortical tubules from fasted rats gluconeogenesis was quantified but oxygen uptake was not measured. There are other investigations, however, in which oxygen uptake was measured in livers from fed rats in addition to parameters such as glycogenolysis and Ca^{2+} fluxes [34,35]. Various leukotrienes (C4, D4, B4 and E4) and the thromboxane A2 analogue U-46619, for example, inhibit oxygen uptake,

increase glycogenolysis and exert complex effects on cellular Ca^{2+} uptake and release [34]. Another thromboxane A2 analogue, ONO-1113, induced a sustained stimulation of oxygen uptake at low concentrations (5 nM), but was inhibitory at higher concentrations (>10 nM) [33]. These and other observations indicate that the various eicosanoids are able to inhibit or to stimulate oxygen uptake and gluconeogenesis depending on their nature and concentrations. It is thus reasonable to conclude that the action of extracellular NAD^+ is mediated by eicosanoids synthesized in Kupffer and endothelial cells in livers of both fasted and fed rats [13]. Sensitivity to both nordihydroguaiaretic acid and indomethacin suggests participation of prostaglandins, thromboxanes and also leukotrienes. Synergisms and complex time dependencies of release and interactions can be expected so that it is almost impossible to attribute each individual effect to a given group of eicosanoids.

NAD^+ is likely to be locally released [9-12]. This may occur via connexin 43 in non-parenchymal liver cells, including Kupffer cells, stellate cells and endothelial cells [9-11] or in consequence of cell lysis in areas of inflammation and tissue injury [12]. Furthermore, evidence obtained in a previous work reveals that extracellular NAD^+ is rapidly transformed along the hepatic acinus and that most of its metabolic effects in livers from fed rats are heterogeneously distributed over the liver parenchyma [14]. Data of the present work suggest that the action on gluconeogenesis is also unequally distributed over the liver parenchyma. The observation that inhibition of oxygen uptake and gluconeogenesis was practically absent in retrograde perfusion indicates that the signal or the signalling agents which produce inhibition are generated in pre-sinusoidal regions. Even if these signals are generated in retrograde perfusion, they cannot not reach the hepatocytes due to the opposite flow direction. This observation supports the proposition that the action of extracellular NAD^+ will be different depending on the site of its release. For example, if NAD^+ is locally released in the perivenous region, stimulation of both oxygen uptake and gluconeogenesis are to be expected. Much work is still needed to clarify the exact role of extracellular NAD^+ in the liver and other tissues but it seems likely that this compound and/or its metabolites ADP-ribose and cyclic ADP-ribose, in conjunction with adenosine, ATP and eicosanoids, play a paracrine role in the regulation of localized cellular activities.

Acknowledgements

This work was supported by a grants from the Programa Nacional de Núcleos de Excelência (PRONEX) and from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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Table 1

Peak changes in glucose production and oxygen uptake as a function of the extracellular NAD^+ concentration. The data were obtained from the experimental results shown in Figure 1. The peak decreases were calculated from the rates of glucose production and oxygen uptake after 20 minutes lactate infusion (F_L) and the corresponding minimal rates (F_{-peak}) during NAD^+ infusion, i.e., peak decrease = $F_{-peak} - F_L$. The peak increases were calculated from the rates of gluconeogenesis and oxygen uptake after 20 minutes lactate infusion (F_L) and the corresponding maximal rates (F_{+peak}) during NAD^+ infusion, i.e., peak increase = $F_{+peak} - F_L$. The *p* (probability) values were obtained by applying one way variance analysis (ANOVA) to the data.

Inhibition	**Stimulation**	**Portal NAD^+ concentration (μM)**			***p*/ mean ratios**
(μmol min^{-1} g^{-1})		25 (n = 6)	50 (n = 6)	100 (n = 4)	
Glucose: peak decrease		−0.17±0.04	−0.53±0.10	−0.69±0.03	< 0.001
Oxygen: peak decrease		−0.36±0.06	−0.93±0.11	−1.39±0.17	< 0.001
$\frac{\Delta\text{oxygen}}{\Delta\text{glucose}}$		2.12	1.75	2.01	**1.96±0.11**
	Glucose: peak increase	0.28±0.03	0.30±0.02	0.14±0.03	0.004
	Oxygen: peak increase	0.32±0.06	0.40±0.05	0.16±0.04	0.042
	$\frac{\Delta\text{oxygen}}{\Delta\text{glucose}}$	1.14	1.34	1.14	**1.21±0.07**

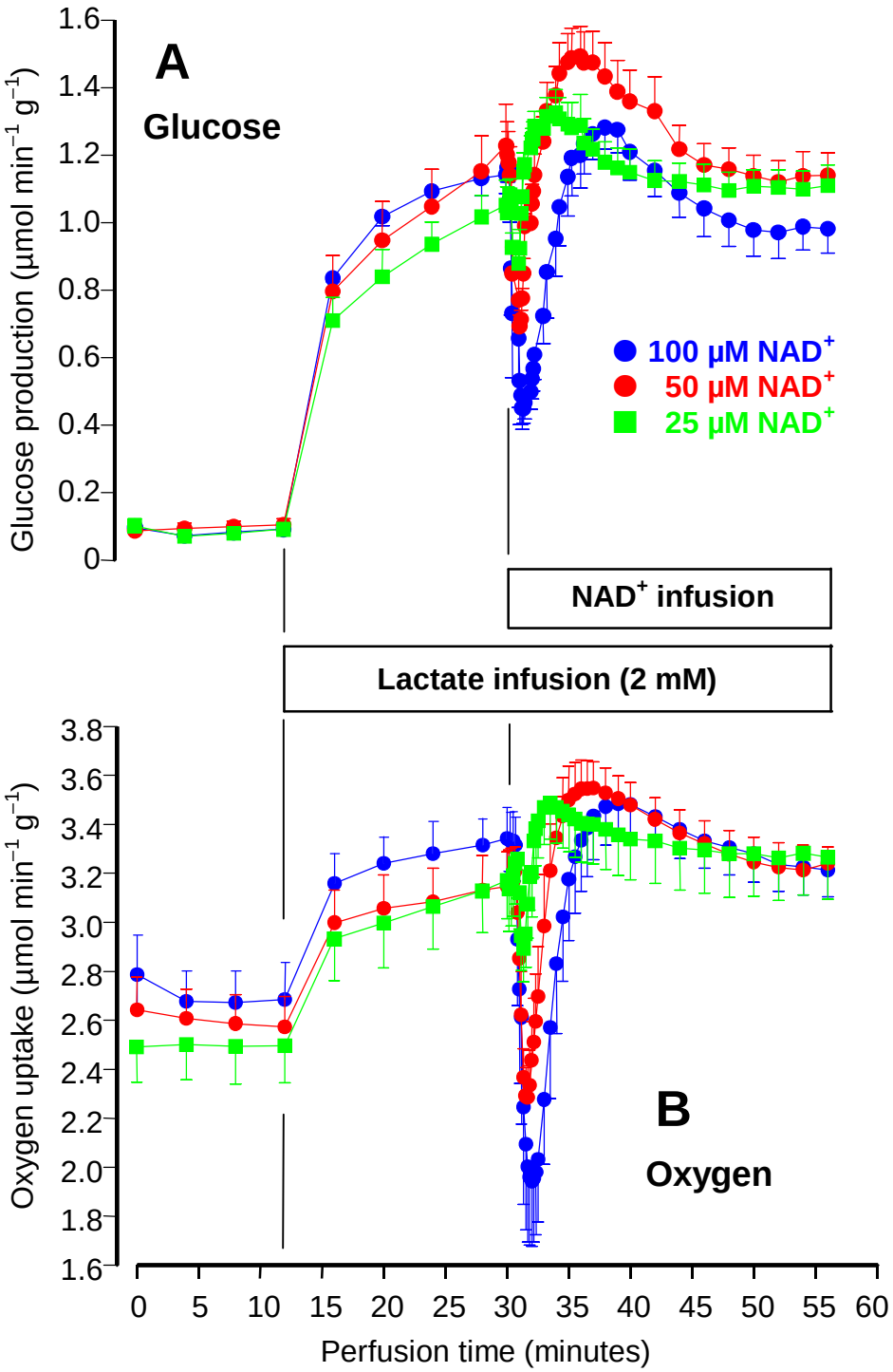

Figure 1. **Time courses of the actions of several NAD^+ concentrations on glucose production (A) and oxygen uptake (B) in the perfused rat liver.** Livers from 24-hours fasted rats were perfused in the antegrade mode as described in Materials and methods. After oxygen uptake stabilization, lactate and NAD^+ (at the indicated concentrations) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means plus mean standard errors of 4-6 liver perfusion experiments for each NAD^+ concentration.

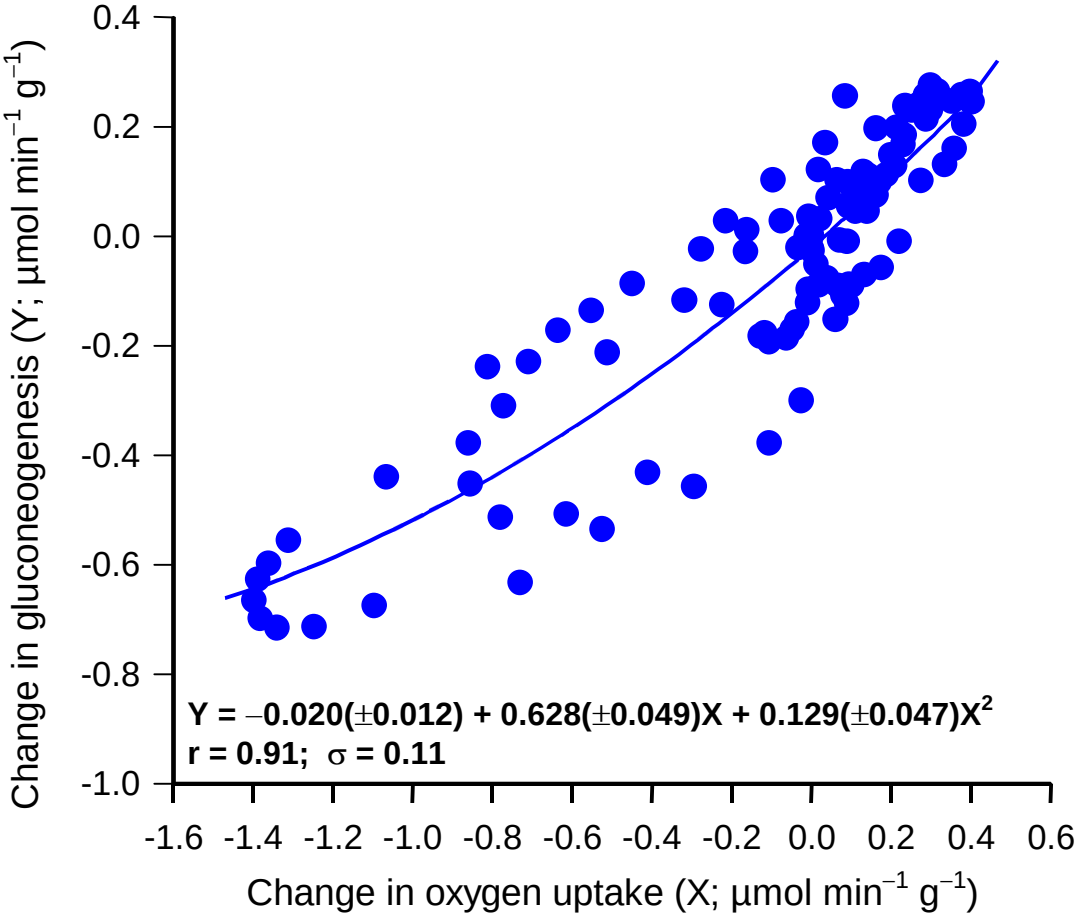

Figure 2. **Changes in gluconeogenesis (Y) versus changes in oxygen uptake (X) due to NAD^+ infusion at various concentrations (25, 50 and 100 µM).** Data were obtained from the time-course relationships shown in Figure 1. The changes in gluconeogenesis were calculated as $Y = (G - G_L)$, G_L being the gluconeogenesis rates in the presence of lactate at 30 minutes perfusion time and G the gluconeogenesis rates in the presence of NAD^+ + lactate. Similarly, the changes in oxygen uptake were calculated as $X = (R - R_L)$, R_L being the oxygen uptake rates in the presence of lactate at 30 minutes perfusion time and R the oxygen uptake rates in the presence of NAD^+ + lactate. The continuous line corresponds to the parabolic regression curve obtained by means of a least-squares fit.

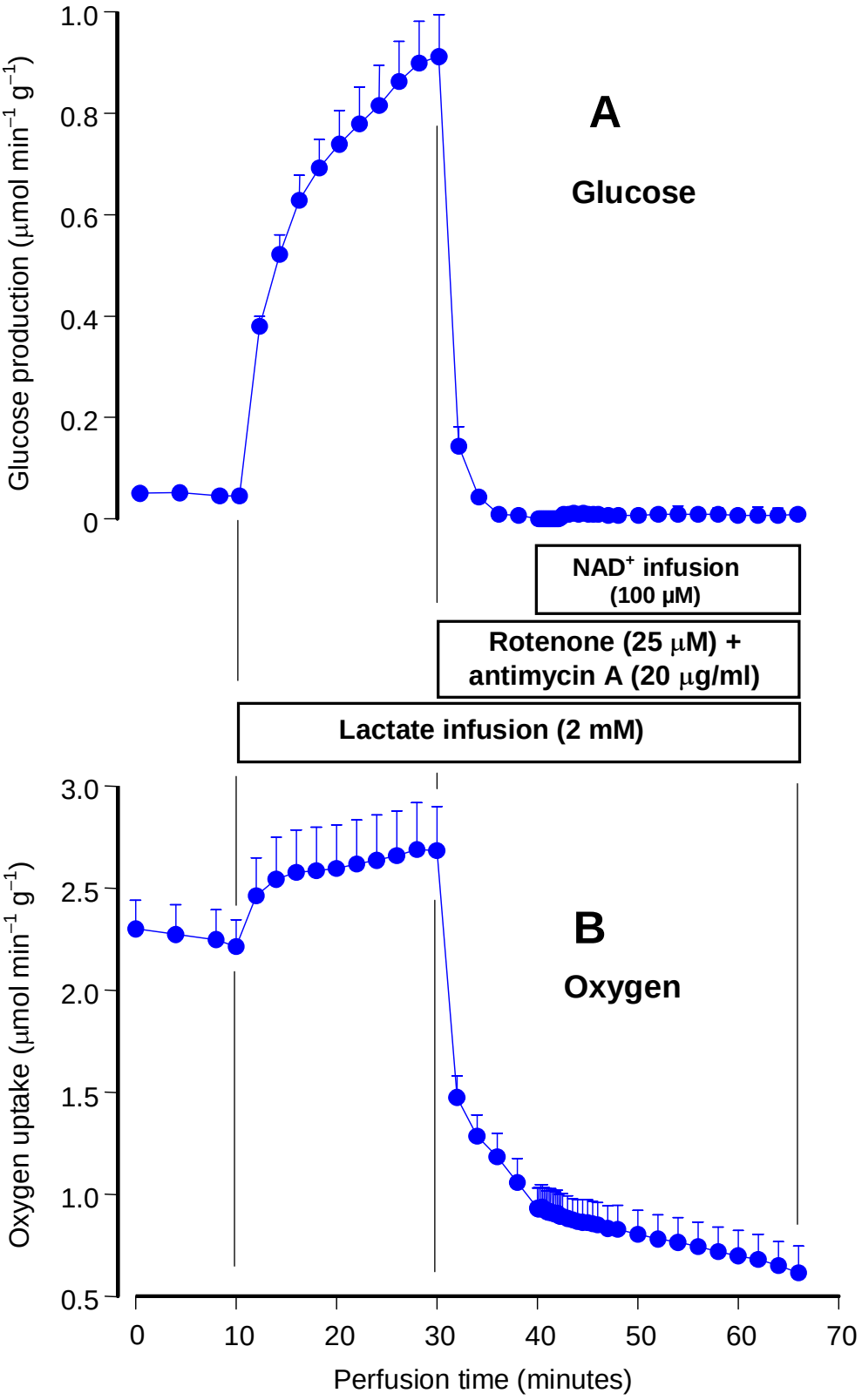

Figure 3. **Changes in glucose production (A) and oxygen uptake (B) caused by sequential and cumulative infusions of lactate, rotenone + antimycin A and NAD^+ into the portal vein.** Livers from fasted rats were perfused in the antegrade mode as described in Materials and Methods. Samples of the effluent perfusate were collected for glucose assay. Oxygen in the effluent perfusion fluid was monitored polarographically. Data are means ± mean standard errors of 3 liver perfusion experiments.

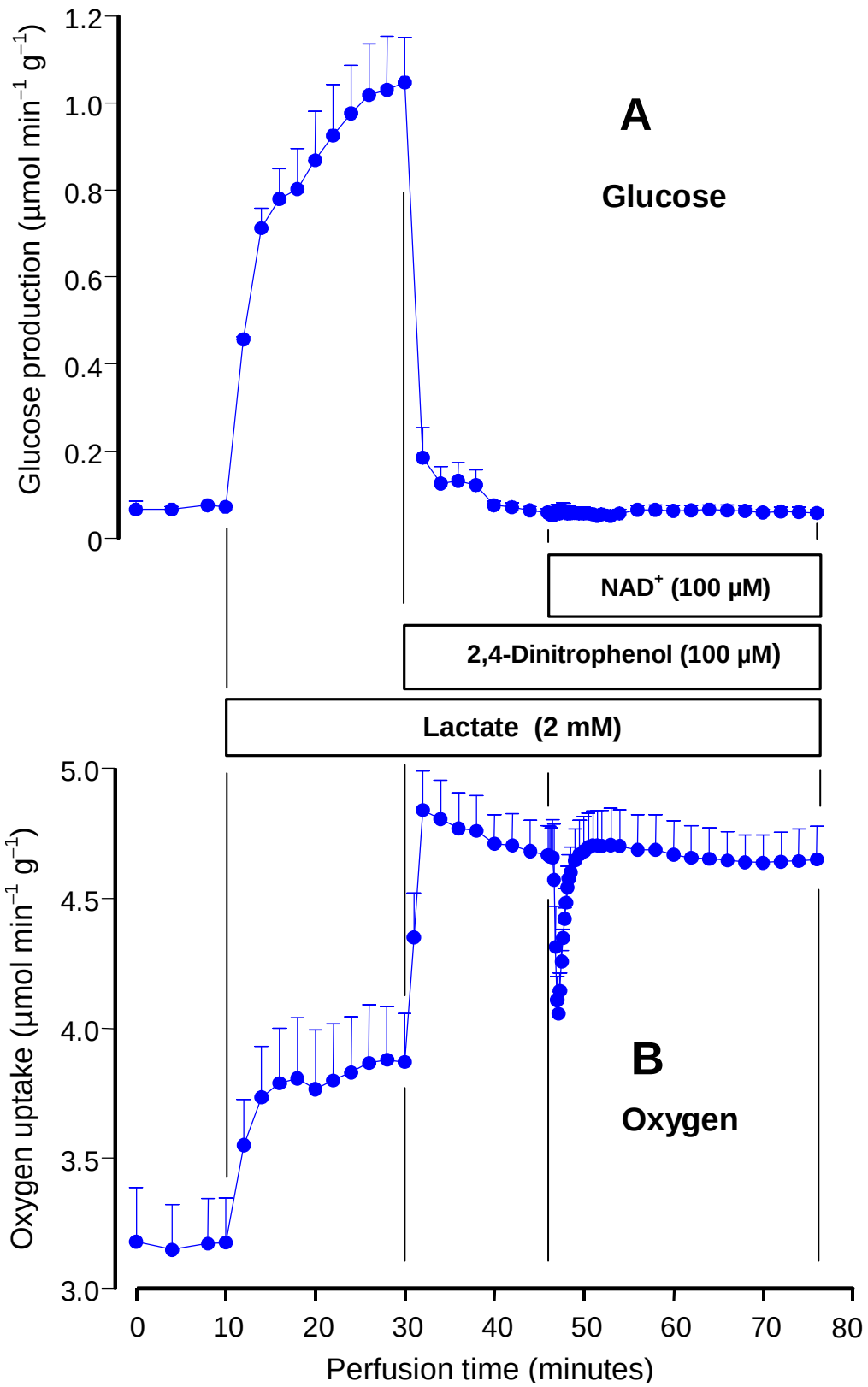

Figure 4. **Changes in glucose production (A) and oxygen uptake (B) caused caused by sequential and cumulative infusions of lactate, 2,4-dinitrophenol and NAD^+.** Livers from fasted rats were perfused in the antegrade mode as described in Materials and methods. Samples of the effluent perfusate were taken for glucose assay. Oxygen in the effluent perfusion fluid was monitored polarographically. Data are means ± mean standard errors of 3 liver perfusion experiments.

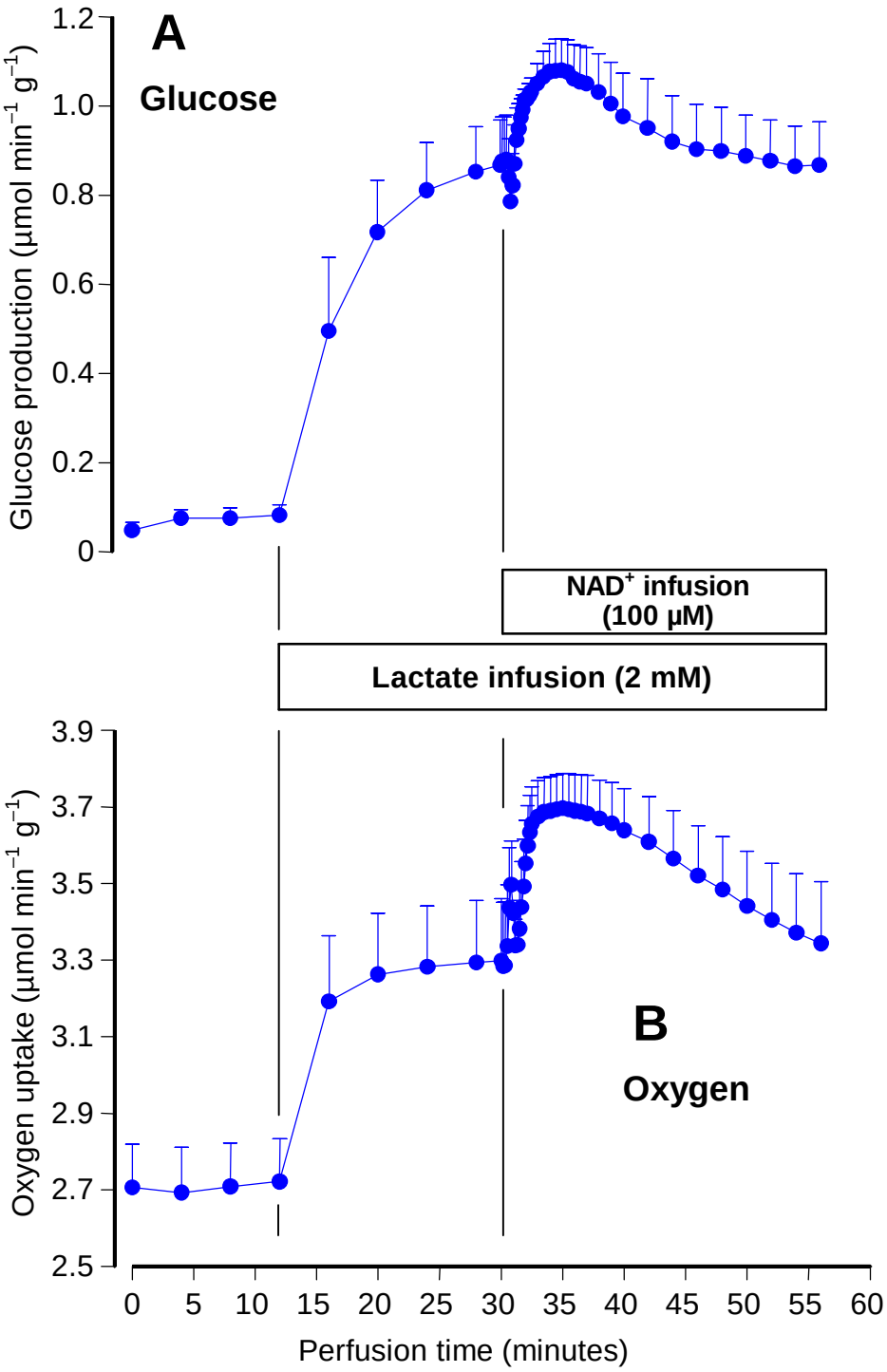

Figure 5. **Changes in glucose production (A) and oxygen uptake (B) caused by NAD^+ infusion into the hepatic vein in retrograde perfusion.** Livers from fasted rats were perfused in the retrograde mode (hepatic vein → portal vein) as described in Materials and Methods. Samples of the effluent perfusate were taken for glucose assay. Oxygen in the effluent perfusion fluid was monitored polarographically. Data are means ± mean standard errors of 5 liver perfusion experiments.

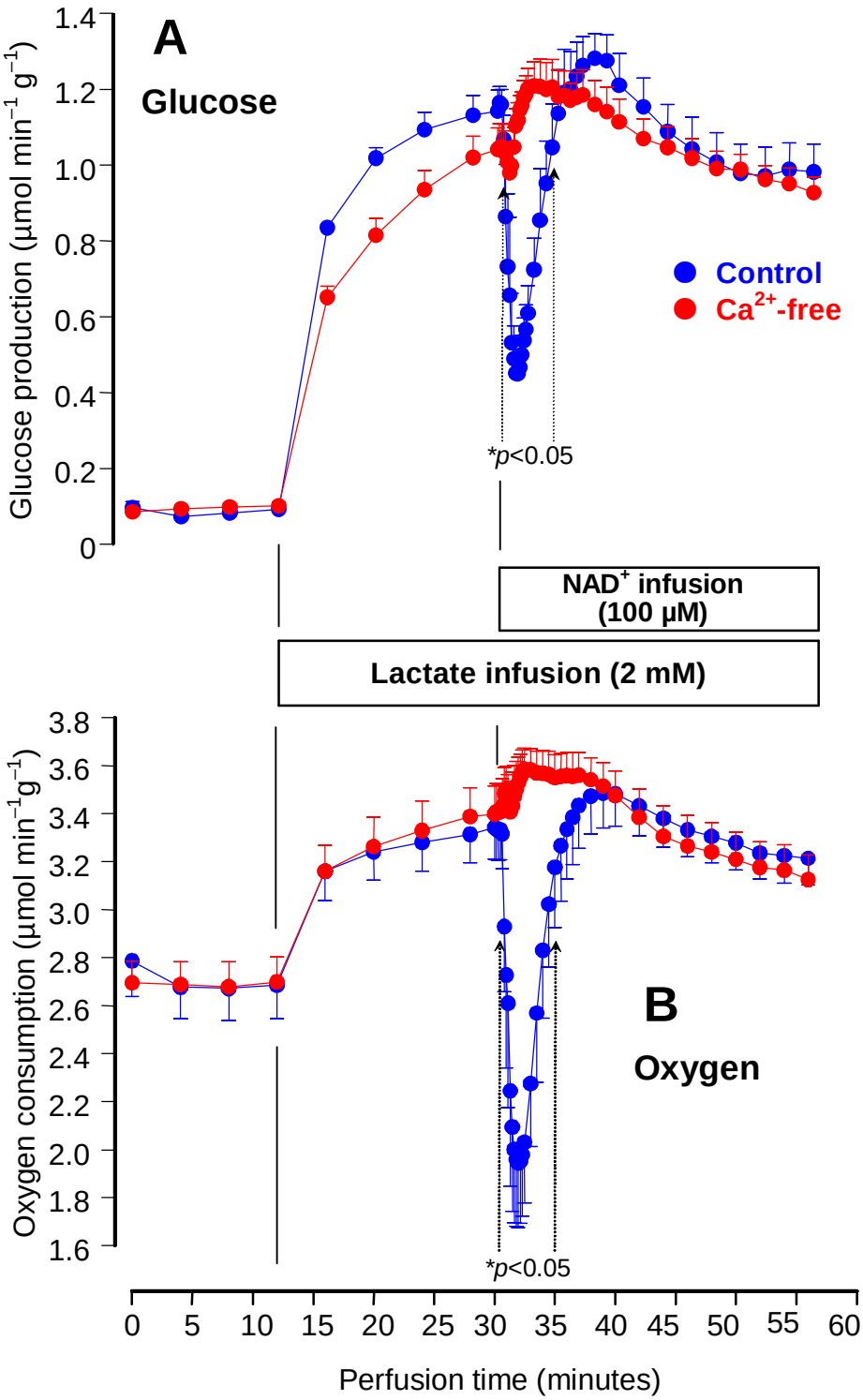

Figure 6. **Influence of Ca^{2+}-free perfusion on the actions of NAD^+ on glucose production (A) and oxygen uptake (B) in the perfused rat liver.** Livers from fasted rats were perfused in the antegrade mode as described in Materials and Methods. Ca^{2+}-free perfusion was preceded by the depletion of the cellular stores. After oxygen uptake stabilization, lactate (2 mM) and NAD^+ (100 µM) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means ± mean standard errors of 5 liver perfusion experiments for each condition. Statistical significance of the differences between control (●) and Ca^{2+}-free perfusion (●) was evaluated by means of multivariate variance analysis (MANOVA). The time spans presenting significant differences ($p < 0.05$) are indicated by arrows.

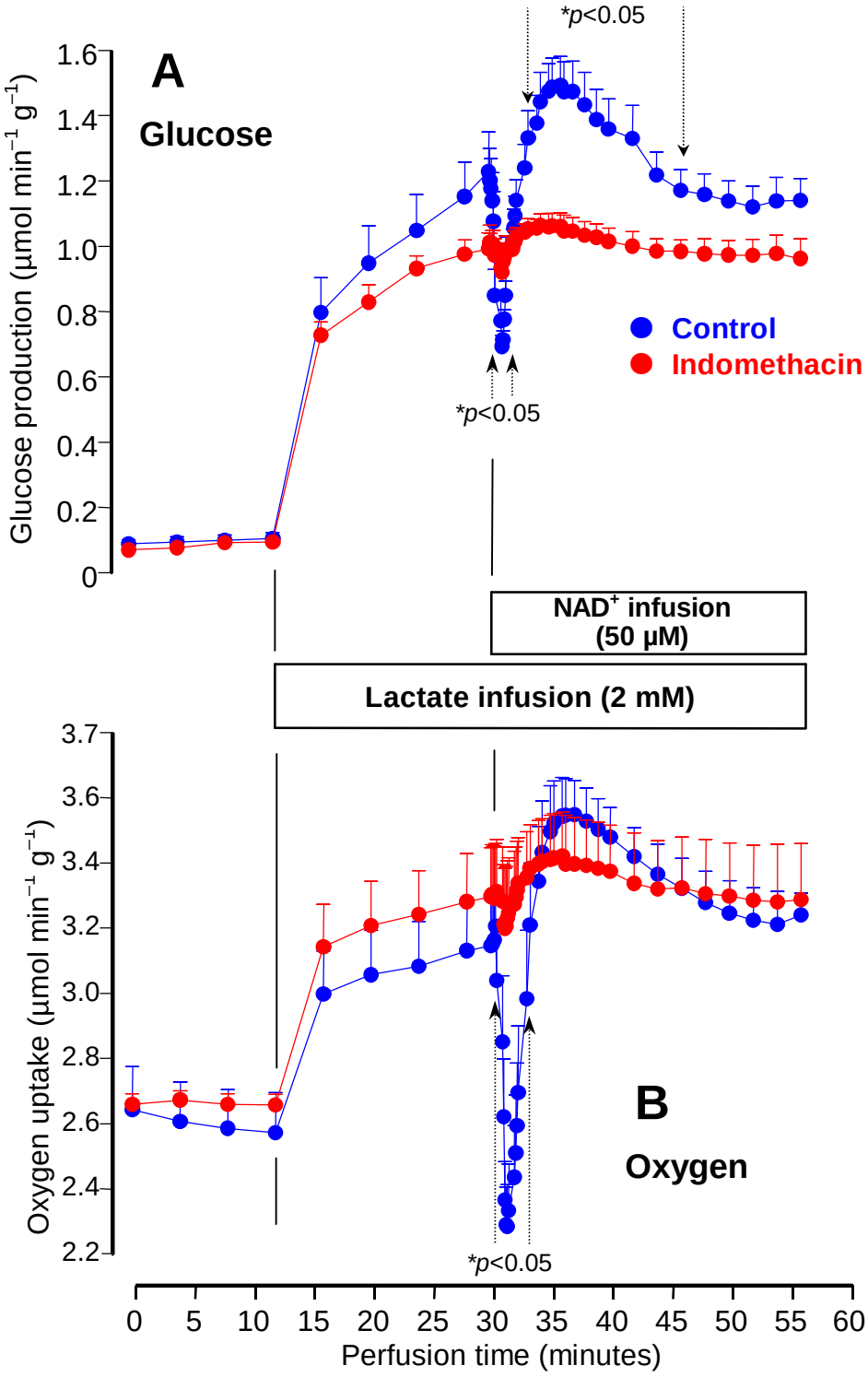

Figure 7. **Influence of indomethacin (30 µM) on the actions of NAD$^+$ on glucose production (A) and oxygen uptake (B) in the perfused rat liver.** Livers from fasted rats were perfused in the antegrade mode as described in Materials and Methods. In one series (●) indomethacin was introduced at time zero. Lactate (2 mM) and NAD$^+$ (50 µM) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means ± mean standard errors of 5 liver perfusion experiments for each condition. Statistical significance of the differences between control (●) and presence of indomethacin (●) was evaluated by means of multivariate variance analysis (MANOVA). The time spans presenting significant differences ($p < 0.05$) are indicated by arrows.

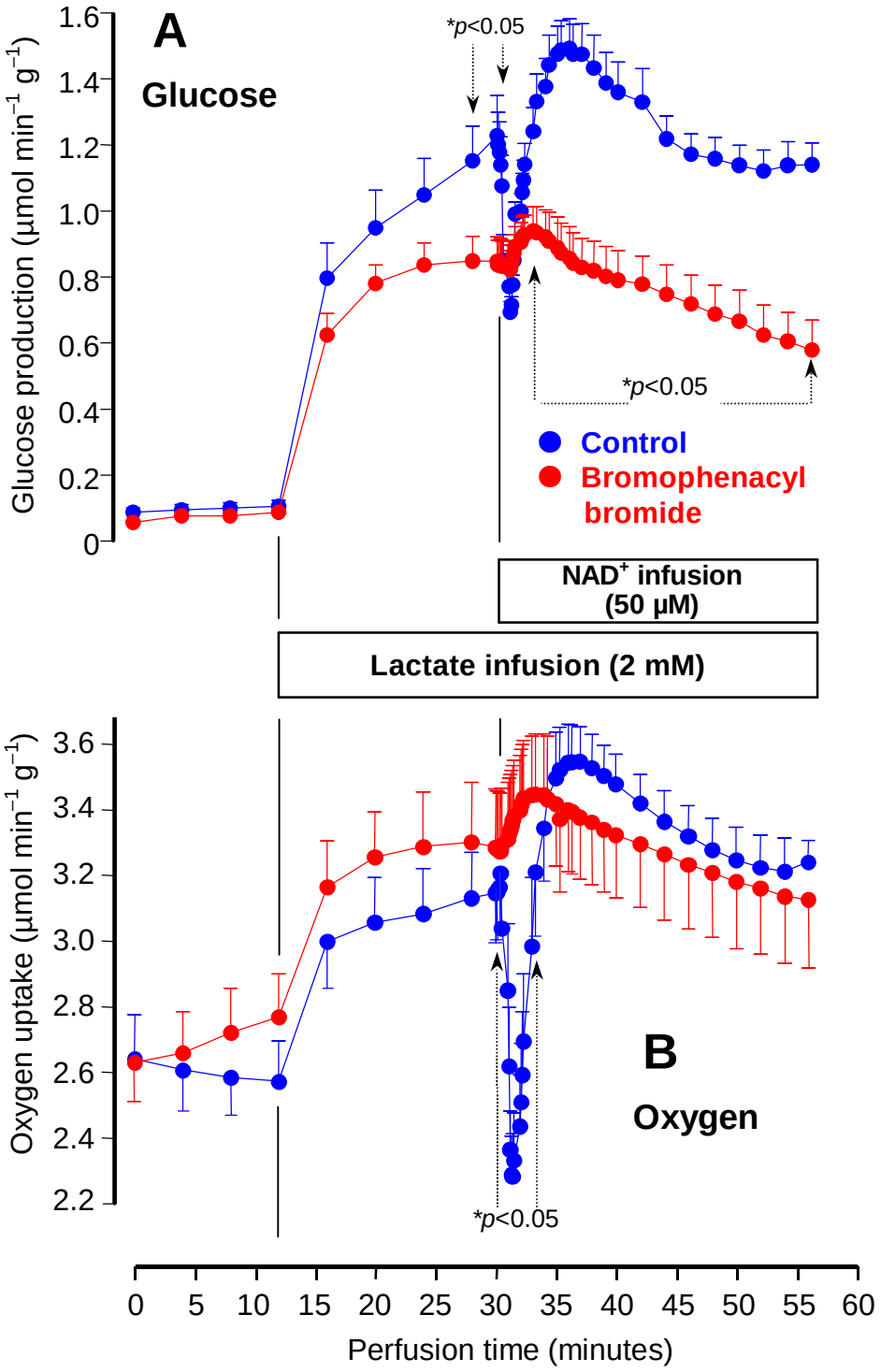

Figure 8. **Influence of bromophenacyl bromide on the actions of NAD^+ on glucose production (A) and oxygen uptake (B) in the perfused rat liver.** Livers from fasted rats were perfused in the antegrade mode as described in Materials and Methods. In one series (●) bromophenacyl bromide (100 µM) was introduced at time zero. Lactate (2 mM) and NAD^+ (50 µM) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means ± mean standard errors of 5 liver perfusion experiments for each condition. Statistical significance of the differences between control (●) and presence of bromophenacyl bromide (●) was evaluated by means of multivariate variance analysis (MANOVA). The time spans presenting significant differences ($p < 0.05$) are indicated by arrows.

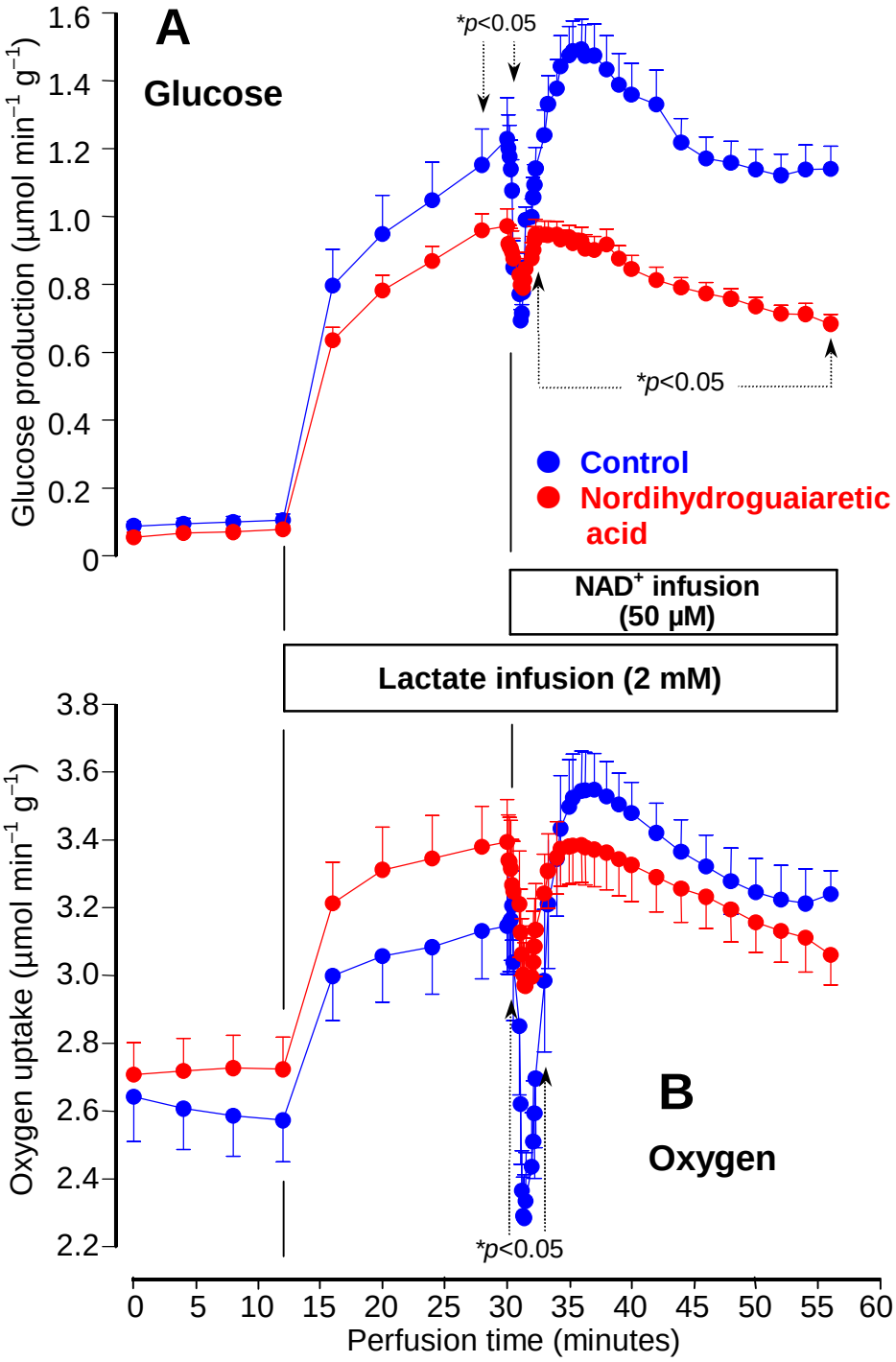

Figure 9. **Influence of nordihydroguaiaretic acid on the actions of NAD^+ on glucose production (A) and oxygen uptake (B) in the perfused rat liver.** Livers from fasted rats were perfused in the antegrade mode as described in Materials and Methods. In one series (●) nordihydroguaiaretic acid (25 µM) was introduced at time zero. Lactate (2 mM) and NAD^+ (50 µM) were infused at the indicated times. Samples of the effluent perfusate were collected for glucose assay. Oxygen was monitored polarographically. Data represent the means ± mean standard errors of 5 liver perfusion experiments for each condition. Statistical significance of the differences between control (●) and presence of nordihydroguaiaretic acid (●) was evaluated by means of multivariate variance analysis (MANOVA). The time spans presenting significant differences ($p < 0.05$) are indicated by arrows.

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