Influência da artrite induzida por adjuvante sobre alguns parâmetros modificados pelo NAD^+ extracelular no fígado de ratos Holtzman

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RESUMO

INTRODUÇÃO E OBJETIVOS — As alterações no perfil hormonal e de citocinas durante a artrite induzem o estabelecimento de um quadro inflamatório crônico no organismo afetado. As interações complexas entre citocinas e fatores de crescimento, que são responsáveis por muitos dos sintomas associados à síndrome, são parcialmente mediadas por eicosanóides. Os eicosanóides modulam a função do hepatócito de modo parácrino e agem, por exemplo, sobre a glicogênese e a glicogenólise através dos seus efeitos sobre a atividade e a expressão de várias enzimas. Em adição à produção de eicosanóides, o quadro inflamatório crônico induzido pela artrite poderia também afetar a expressão de vários tipos de receptores dos eicosanóides. Por todos estes motivos, parece razoável esperar uma resposta modificada do fígado de animais artríticos a efetores metabólicos cuja ação seja mediada por eicosanóides. Um agente deste tipo é o NAD^+ extracelular, que pode estar presente em áreas localizadas, durante a inflamação graças a extrusões catalisadas pela conexina 43 e também em decorrência da lise celular. Sua ação tem sido investigada no fígado de rato em perfusão onde ele produz aumentos na pressão portal e arterial, induz movimentos de Ca^{2+}, causa aumento transitório da glicogenólise e inibição transitória da produção de glicose seguida de estimulação. Estas ações são consequência de uma interação entre células parenquimais e não-parenquimais mediada por eicosanóides. Esta interação, combinada com a estreita associação entre a artrite e a produção de eicosanóides, faz surgir a hipótese de que esta síndrome poderia afetar a ação do NAD^+ extracelular no fígado. O objetivo do presente trabalho foi justamente o de investigar esta questão. Ratos Holtzman portadores de artrite induzida pelo adjuvante de Freund foram utilizados como modelo de caquexia e três parâmetros metabólicos foram medidos sob diferentes condições no fígado em perfusão: as produções de glicose e piruvato a partir de lactato e o consumo de oxigênio. Adicionalmente, foi também medida a pressão portal de perfusão. Ratos Holtzman foram utilizados porque esta cepa responde de modo mais estável ao adjuvante de Freund e também com propósitos comparativos, já que os efeitos do NAD^+ extracelular sobre o metabolismo hepático foram investigados até agora apenas em ratos Wistar. Os resultados deverão proporcionar uma primeira avaliação da influência da artrite sobre a ação hepática de um agente parácrino típico.

MÉTODOS — Ratos machos Holtzman, pesando de 190 a 230 g e alimentados com ração padronizada (Nuvilab®) foram utilizados. Para a indução da artrite, 100 µl do adjuvante de Freund, suspenso em óleo mineral em concentração de 0,5% (w/v), foram injetados na pata traseira esquerda. Animais com as lesões características aos 14 dias após a injeção do adjuvante foram selecionados para os experimentos.

O fígado isolado foi perfundido no modo não-recirculante nos modos anterógrado (veia porta → veia hepática) ou retrógrado (veia hepática → veia porta). O líquido de perfusão foi tampão Krebs/Henseleit-bicarbonato (pH 7,4), saturado com O_2 e CO_2 (95:5) através de um oxigenador de membrana e aquecido a 37°C. Fígados de ratos em jejum de 24 horas foram utilizados. Os procedimentos cirúrgicos de canulação e isolamento foram feitos sob anestesia de tiopental sódico (50 mg/kg).

Glicose e piruvato foram medidos enzimaticamente no perfusado efluente. A concentração de oxigênio foi medida polarograficamente. A pressão portal foi medido através de um transdutor de pressão. Perfusão livre de cálcio foi feita usando líquido livre de Ca^{2+} e após depleção dos estoques celulares de Ca^{2+}.

RESULTADOS — 1) A pressão portal de perfusão foi aumentada transitoriamente por NAD^+ 100 µM em fígados de ratos controle e artríticos com incrementos de pico de 3,15±0,28 e 4,40±0,18 mm Hg, respectivamente.

2) A infusão de lactato 2 mM em perfusão anterógrada (veia porta → veia hepática) produziu aumentos aproximadamente estáveis nas produções de glicose e piruvato e no consumo de oxigênio. Na condição de artrite estes incrementos foram menos pronunciados. A introdução de NAD^+ produziu uma inibição transitória de todos os parâmetros, seguida de uma recuperação. Na condição controle a recuperação foi seguida por um estímulo transitório no caso da produção de glicose e do consumo de oxigênio. A fase inibitória na condição de artrite foi caracterizada por retardos nos tempos de pico e por tempos mais longos para atingir 50% de recuperação. Em termos absolutos os decréscimos de pico causados pelo NAD^+ na condição de artrite foram similares aos controles; em termos relativos, no entanto, os decréscimos na produção de glicose e consumo de oxigênio foram mais pronunciados na condição de artrite.

3) A infusão de lactato 2 mM em perfusão retrógrada (veia hepática → veia porta) produziu aumentos na produção de glicose e consumo de oxigênio os quais foram similares àqueles encontrados em perfusão anterógrada. Os efeitos do NAD^+ 100 μM foram estímulos transitórios na produção de glicose e consumo de oxigênio e inibição transitória na produção de piruvato. Os estímulos da produção de glicose e do consumo de oxigênio foram menos pronunciados na condição de artrite.

4) A perfusão livre de Ca^{2+} praticamente aboliu as ações do NAD^+ nas duas condições, controle e artrite.

5) A infusão portal de indometacina 30 μM (inibidor da cicloxigenase) reduziu fracamente a produção de glicose antes da infusão do NAD^+, sem afetar o consumo de oxigênio e a produção de piruvato. As ações do NAD^+ sobre o consumo de oxigênio e a produção de glicose foram praticamente abolidas pela indometacina e aquelas sobre a produção de piruvato foram fortemente reduzidas.

6) A infusão portal de brometo de bromofenacila 100 μM (inibidor da fosfolipase A_2) eliminou as inibições da produção de glicose e do consumo de oxigênio pelo NAD^+ nas duas condições, controle e artrite. Estímulos transitórios dos dois parâmetros foram bem evidentes; eles tenderam a ser menos pronunciados na condição de artrite. A produção de piruvato continuou sendo inibida pelo NAD^+ na presença de brometo de bromofenacila, mas em menor extensão.

7) A infusão portal do ácido nordihidroguaiarético (inibidor da lipoxigenase) a uma concentração de 25 μM, diminuiu a ação estimuladora do NAD^+ sobre a produção de glicose e o consumo de oxigênio na condição controle e prolongou a ação inibidora, especialmente na condição de artrite.

CONCLUSÕES E DISCUSSÃO — 1) A resposta do fígado de ratos Holtzman ao NAD^+ portal (perfusão anterógrada) difere daquela do fígado de ratos Wistar no fato de que as fases inibidoras da neoglicogênese e do consumo de oxigênio na primeira cepa mostram-se reduzidas no tempo e na extensão e a fase estimuladora que sucede a inibição é mais intensa. Em perfusão retrógrada, porém, na qual estimulação é o único efeito, não há diferença entre ratos Holtzman e Wistar. Esta observação sugere que a estimulação mais intensa em ratos Holtzman em perfusão anterógrada poderia ser consequência da fase inibitória mais curta e menos intensa.

2) A observação de que a inibição da produção de piruvato ocorre mesmo em perfusão retrógrada, onde a inibição do consumo de oxigênio está ausente, sugere um efeito direto do NAD^+ sobre o potencial redox NAD^+-NADH celular.

3) A artrite muda a resposta ao NAD^+ extracelular aumentando (em termos relativos) e prolongando a fase inibitória. Esta fase é sensível a inibidores da síntese de eicosanóides. Por isto, é bem possível que ela seja produzida por eicosanóides liberados por um estímulo causada pelo NAD^+. As inibições mais fortes e mais persistentes da neoglicogênese e do consumo de oxigênio e também o maior aumento na pressão portal na condição de artrite são, portanto, consistentes com uma acentuada capacidade de produzir eicosanóides.

ABSTRACT

INTRODUCTION AND AIMS — The alterations in the profile of hormones and cytokines during arthritis induce the establishment of a chronic inflammatory picture in the affected organism. The complex interactions between cytokines and growth factors, which are responsible for many of the symptoms associated to the disease, are partly mediated by eicosanoids. The eicosanoids modulate the function of the hepatocyte in a paracrine way and they act, for example, on glycogenesis and glycogenolysis through their effects on the activity and expression of several enzymes. In addition to the production of eicosanoids the chronic inflammatory picture which characterizes arthritis could also be affecting the expression of the various types of eicosanoid receptors. For all these reasons it seems plausible to expect a modified response of the liver of arthritic animals to metabolic effectors whose action is mediated by eicosanoids. One such agent is extracellular NAD^+ which is believed to arise in localized areas during inflammation in consequence of tissue injury and cell lysis and of extrusions catalyzed by connexin 43. Its action has been investigated in the perfused rat liver where it causes increases in portal and arterial pressure, induces Ca^{2+} movements, causes transient increases in glycogenolysis and transient inhibition of glucose production followed by stimulation. There is ample evidence that these actions are consequence of an interaction between parenchymal and non-parenchymal cells via eicosanoids. This notion, when combined with the intimate association between arthritis and eicosanoid production, leads to the hypothesis that the latter syndrome could be affecting the action of extracellular NAD^+ in the liver. To investigate this question was exactly the purpose of the present work. Rats with adjuvant-induced arthritis were chosen as an experimental model and three metabolic parameters were measured under different conditions in the perfused liver: gluconeogenesis and pyruvate productions from lactate and oxygen uptake. Additionally the perfusion pressure was also measured. Holtzman rats were used because this strain responds in a more stable way to Freund's adjuvant and also for comparative purposes due to the fact that the action of extracellular NAD^+ on hepatic metabolism has been investigated solely in rats of the Wistar strain. The results should allow a first insight into the influence of arthritis on the hepatic action of a typical paracrine agent.

METHODS — Male Holtzman rats, weighing 190-230 g and fed ad libitum with a standard laboratory diet (Nuvilab®) were used. For the induction of adjuvant arthritis, the animals were injected in the left hind paw with 100 µl of Freund's adjuvant, suspended in mineral oil at a concentration of 0.5% (w/v). Animals presenting the characteristic lesions at 14 days after the adjuvant injection were selected for the experiments.

The isolated liver was perfused in the non-recirculating mode in the antegrade (portal vein → hepatic vein) or retrograde directions (hepatic vein → portal vein). The perfusion fluid was the Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with O_2 and CO_2 (95:5) by means of a membrane oxygenator and heated to 37°C. Livers of 24-hours fasted rats were used. The surgical procedures of cannulation and isolation were done under sodium thiopental anesthesia (50 mg/kg).

Glucose and pyruvate were measured enzymatically in the effluent perfusate. The oxygen concentration was measured polarographically. The portal pressure was monitored by means of a pressure transducer. Calcium-free perfusion was done by perfusing with Ca^{2+}-free medium and after depleting the cellular Ca^{2+} pools.

RESULTS — 1) The portal perfusion pressure was transiently increased by 100 µM NAD^+ in both livers of control and arthritic rats with peak increments of 3.15±0.28 and 4.40±0.18 mm Hg, respectively.

2) The infusion of 2 mM lactate into livers in antegrade perfusion (portal vein → hepatic vein) produced relatively stable increases in the productions of glucose and pyruvate and in oxygen uptake. In the arthritis condition these increments were less pronounced. The introduction of NAD^+ produced a transient inhibition of all parameters followed by a recovery. In the control condition the recovery was followed by a transient stimulation in the case of glucose production and oxygen uptake. The inhibitory phase in the arthritis condition was characterized by delayed peak times and longer times for half-maximal recovery. In absolute terms the peak decreases caused by NAD^+ in the arthritis condition were similar to the controls; in relative terms, however, the decreases in glucose production and oxygen uptake in the arthritis condition were more pronounced.

3) The infusion of 2 mM lactate into livers in retrograde perfusion (hepatic vein → portal vein) produced increases in glucose production and oxygen uptake that were similar to those found in antegrade perfusion. The effects of 100 μM NAD^+ were transient stimulations of glucose production and oxygen uptake and transient inhibition of pyruvate production. The stimulations of glucose production and oxygen uptake were both less pronounced in the arthritis condition.

4) Ca^{2+}-free perfusion almost abolished the actions of NAD^+ in both conditions, control and arthritic.

5) Portal indomethacin (30 μM; cicloxygenase inhibitor) slightly reduced glucose production before NAD^+ infusion without affecting oxygen uptake and pyruvate production. The actions of NAD^+ on oxygen uptake and glucose production were almost abolished by indomethacin and those on pyruvate production strongly reduced.

6) Portal bromophenacyl bromide (100 μM; phospholipase A_2 inhibitor) eliminated the inhibitions of glucose production and oxygen uptake by NAD^+ in both conditions, control and arthritis. Transient stimulations of both parameters were apparent instead; they tended to be less pronounced in the arthritis condition. Pyruvate production was still inhibited by NAD^+ in the presence of bromophenacyl bromide, but to a lesser extent.

7) Portal nordihydroguaiaretic acid (inhibitor of lipoxygenase) at the concentration of 25 μM, diminished the stimulatory action of NAD^+ on glucose production and oxygen uptake in the control condition and prolonged the inhibitory action, especially in the arthritis condition.

CONCLUSIONS AND DISCUSSION — 1) The response of the liver from rats of the Holtzman strain to portal NAD^+ (antegrade perfusion) differs from that of the liver from Wistar rats in that the inhibitory phases of gluconeogenesis and oxygen uptake in the former strain are reduced in time and extent and the stimulatory phases which follow inhibition are more pronounced. In retrograde perfusion, however, where stimulation is the only effect, there is no difference between Holtzman and Wistar. This suggests that the more intense stimulation in the former in antegrade perfusion could be consequence of the shorter and less intense inhibitory phases.

2) The finding that inhibition of pyruvate production occurs even in retrograde perfusion, where oxygen uptake inhibition is absent, suggests a direct effect of NAD^+ on the cellular, possibly cytosolic, NAD^+-NADH redox potential.

3) Arthritis changes the response to extracellular NAD^+ by increasing (in relative terms) and prolonging the inhibitory phase and diminishing stimulation. Since the initial inhibition is sensitive to inhibitors of eicosanoid synthesis, it is probable that it is caused by a NAD^+-stimulated eicosanoid release. The stronger and more persistent inhibitions of gluconeogenesis and oxygen uptake and the more accentuated increase in perfusion pressure in the arthritic condition are, thus, consistent with an enhanced capacity of eicosanoid production.

Influence of adjuvant-induced arthritis on the action of extracellular NAD^+ on hepatic gluconeogenesis and related parameters

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Running tittle: Hepatic action of extracellular NAD^+ and arthritis.

Abstract

The influence of arthritis on some metabolic and hemodynamic effects of extracellular NAD^+ was investigated in the perfused liver of Holtzman rats with the additional purpose of comparing the present results with previous results obtained with rats from the Wistar strain. Fasted rats were used and glucose plus pyruvate productions from lactate and the associated oxygen uptake increment were measured as well as the portal portal perfusion pressure. Livers from arthritic rats presented higher transient increments in perfusion pressure upon NAD^+ infusion. The metabolic effects of NAD^+ consisted in initial inhibitions of glucose and pyruvate production and oxygen uptake, followed by recovery and stimulation. In the arthritis condition the inhibitions were more accentuated in relative terms and also more persistent in time, but the subsequent stimulations were smaller or even absent. The inhibitory phase was almost abolished by indomethacin, inhibitor of eicosanoid synthesis, but not by nordihydroguaiaretic acid, an inhibitor of leukotriene synthesis. In comparison with Wistar rats the response to NAD^+ of the liver from Holtzman rats in antegrade perfusion was characterized by a shorter and less intense inhibitory phase and by a more vigorous stimulation. In retrograde perfusion, however, where stimulation was the only effect, there was no difference between both strains. This observation suggests that the more accentuated stimulation in Holtzman rats could be the consequence of a short and less intense inhibitory phase. The stronger and more persistent inhibitions of gluconeogenesis and oxygen uptake as well as the enhanced portal pressure increase in the arthritis condition are consistent with an accentuated capacity of producing eicosanoids by animals under the influence of the arthritis disease.

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

Introduction

The alterations in the profile of hormones and cytokines during arthritis induce the establishment of a chronic inflammatory picture in the affected organism (Meliconi et al., 1988). The complex interactions between cytokines and growth factors, which are responsible for many of the symptoms associated to the disease, are partly mediated by eicosanoids (Lee and Weinblatt, 2001; Pérez et al., 2006). The eicosanoids modulate the function of the hepatocyte in a paracrine way and they act, for example, on glycogenesis and glycogenolysis through their effects on the activity and expression of several enzymes and, additionally, by means of alterations in the sinusoidal blood flow (Püschel and Christ, 1994; Schieferdecker et al., 1999). Furthermore, they modulate the function of the non-parenchymal cells of the liver in paracrine and autocrine ways (Wang et al., 1998). The five most important prostanoids PGD_2, PGE_2, $PGF_2\alpha$, PGI_2 and thromboxane A_2 are produced by Kupffer, endothelial and Ito cells (Fennekohl et al., 1999). The effects related to the eicosanoids that are present in the circulation or in the interstitial fluid are the result of their interactions with heptahelical transmembrane receptors whose secondary messengers are cAMP or IP_3-Ca^{2+} (Fennekohl et al., 1999). In addition to the production of eicosanoids the chronic inflammatory picture of arthritis could also be affecting the expression of the various types of eicosanoid receptors (Picoletti et al., 1991; Decker, 1998; Pérez et al., 2004).

For all these reasons it seems reasonable to expect a modified response of the liver of arthritic animals to metabolic effectors whose action is mediated by eicosanoids. To this group of effectors belong the purinergic agents, e.g., extracellular adenosine, AMP and ATP (Fernandes et al., 2002), and extracellular NAD^+ (Broetto-Biazon et al., 2004). Extracellular NAD^+ is believed to arise in localized areas during inflammation in consequence of tissue injury and cell lysis and also in consequence of extrusions catalized by connexin 43 (Bruzzone et al., 2000; Ohlrogge et al., 2002). Its action has been investigated in the perfused rat liver (Broetto-Biazon et al., 2004; Gimenes et al., 2006; Martins et al., 2006). The main effects that were observed are: a) increases in portal and arterial pressure; b) induction of Ca^{2+} movements suggesting increased cytosolic Ca^{2+} levels; c) initial inhibition of oxygen consumption followed by stimulation; d) transient increases in glycogenolysis; e) initial inhibition of glucose production followed by stimulation. These actions are not uniformly distributed over the liver parenchyma. 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 (Gimenes et al., 2006; Martins et al., 2006). Most of these effects are Ca^{2+}-dependent and most of them seem to result from an interaction between parenchymal and non-parenchymal cells via eicosanoid production. The latter conclusion is based mainly on the observation that the action of extracellular NAD^+ on the liver functions is sensitive to inhibitors of eicosanoid synthesis (Broetto-Biazon et al., 2004; Martins et al., 2006). The metabolic and Ca^{2+} mobilizing actions of extracellular NAD^+ seem to be intimately connected to its enzymic transformation catalyzed by a bifunctional enzymatic system (NAD^+ glycohydrolase/ADP-ribosyl-cyclase), which produces ADP-ribose, free nicotinamide, nucleotide nicotinic acid-adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose (cADPR) (Lee et al., 1994; Ziegler, 2000; Chini et al., 2002). The activity of this enzymic system has been shown to increase during thioacetamide induced cirrhosis (Gan et al., 2005), an observation that additionally strenghtens the hypothesis of a possible connection between the action of extracellular NAD^+ in the liver and its inflammatory status.

Taking thus into account the points raised above, the present work was planned to investigate the possible influence of arthritis on the action of extracellular NAD^+ on some metabolic and functional parameters in the isolated perfused rat liver. Rats with adjuvant induced arthritis were chosen as the experimental model (Pearson, 1956). In the present investigation, the experimental efforts were concentrated on gluconeogenesis from lactate and the corresponding changes in oxygen uptake, two parameters that have been shown to respond in an almost synchronous mode to extracellular NAD^+ (Martins et al., 2006). Holtzman rats were used because this strain responds in a stable way to Freund's adjuvant (Bersani-Amado et al., 1990; Kelmer-Bracht et al., 2003) whereas the response of Wistar rats presents considerable fluctuations among individuals (Banik et al., 2002). Furthermore, the use of Holtzman rats will also be useful for comparative purposes taking into account the fact that the action of extracellular NAD^+ on hepatic metabolism has been investigated solely in rats of the Wistar strain (Broetto-Biazon et al., 2004; Gimenes et al., 2006; Martins et al., 2006) and there is evidence that Holtzman and Wistar rats may respond differently when eicosanoids are involved (Weinberg et al., 1997). The results should also allow a first insight into the influence of arthritis on the hepatic action of a typical paracrine agent.

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 (Holtzman), weighing 190-230 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 (Scholz and Bücher, 1965). 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 $\rightarrow$ hepatic vein) was performed. In some selected experiments, however, retrograde perfusion (hepatic vein $\rightarrow$ 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. 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 (Reinhart et al., 1982), this procedure depletes the intracellular Ca^{2+}-pools which are normally mobilized when Ca^{2+}-agonists are infused.

Animals and treatments

Male Holtzman rats, weighing 190–230 g, were fed *ad libitum* with a standard laboratory diet (Nuvilab®). For the induction of adjuvant arthritis, the animals were injected in the left hind paw with 100 µl of Freund's adjuvant (heat inactivated *Mycobacterium tuberculosis*, derived from human strains H37Rv), suspended in mineral oil at a concentration of 0.5% (w/v). Animals showing the characteristic lesions at 14 days after adjuvant injection were selected for the experiments (Pearson, 1956). Rats of similar ages (60 days) were injected with mineral oil and served as controls.

Analytical

The oxygen concentration in the effluent perfusate was monitored continuously, employing a teflon-shielded platinum electrode (Scholz and Bücher, 1965). Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their glucose (Bergmeyer and Bernt, 1974) and pyruvate (Czok and Lamprecht, 1974) contents.

Perfusion pressure

The portal perfusion pressure was monitored by means of a pressure transducer (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany). The sensor was positioned near to the entry vessel (portal vein) and the transducer was connected to a recorder. The pressure changes were computed from the recorder tracings and expressed as mm Hg.

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). Two-way variance analyses with post-hoc testing were done for comparing more than two means. The results are mentioned in the figures and tables as the *p* values.

Results

In the first experiments the changes in portal perfusion pressure were monitored in perfused livers from control and adjuvant-induced arthritic Holtzman rats. The time-courses of the mean pressure changes are shown in Figure 1. Livers from arthritic rats tended to offer higher resistance to perfusion during basal conditions. The introduction of lactate did not produce any change in the portal perfusion pressure (not shown). The introduction of NAD^+, however, produced transient increases in the perfusion pressure of both livers of control and arthritic rats. Both visual inspection of the curves in Figure 1 and the data in Table 1 reveal a significantly higher peak-increment in the arthritic condition. The peak time was not significantly different in the arthritic condition, the same being valid for the time for half-maximal decay ($t_{½}$). The latter was computed by numerical interpolation and it corresponds to the difference between the peak time and the time at which the peak increment experienced a 50% reduction. It can be regarded as a measure of the persistence of the NAD^+ effects.

The influence of arthritis and NAD^+ on gluconeogenesis from 2 mM lactate in antegrade perfusion (portal vein $\rightarrow$ hepatic vein) is illustrated by Figure 2. The changes in oxygen uptake and pyruvate production, two parameters strictly related to gluconeogenesis are also shown. The infusion of lactate increased all parameters to new steady-states. Oxygen uptake and glucose production were less increased in the arthritis condition, but pyruvate was similarly increased in both conditions. The mean difference between gluconeogenesis of the control and arthritic condition was 49%. The introduction of 100 μM NAD^+ produced transient inhibitions of all parameters followed by a recovery. For oxygen uptake and gluconeogenesis this phenomenon has already been described in livers from Wistar rats (Martins et al., 2006). The rapid recoveries of glucose production and oxygen uptake in the control condition were followed by clear stimulations. The peak increments in glucose production and oxygen uptake were, respectively, 0.276$\pm$0.047 and 0.365$\pm$0.058 µmol min^{-1} g^{-1}. No stimulation following the recovery was found in the arthritis condition. Besides this, other characteristics of the response in the arthritis condition were (a) delayed peak times and (b) considerably longer times for half-maximal recovery, as revealed by Table 2. These phenomena were found for all three variables and the differences were especially pronounced in the times for half-maximal recovery ($t_{½}$) of glucose production and oxygen uptake. Table 2 also

allows to compare the peak decreases in both absolute and relative terms. The absolute peak decreases in the arthritis condition were not significantly different from those in the control condition, although a strong tendency toward a greater peak decrease in oxygen uptake should be remarked. In relative terms, however, the peak decreases in glucose production and oxygen uptake were clearly more pronounced in the arthritis condition.

It has been shown in previous investigations with livers from healthy Wistar rats that changing the perfusion direction (hepatic vein $\rightarrow$ portal vein instead of portal vein $\rightarrow$ hepatic vein) practically eliminates the initial inhibitions of oxygen uptake and gluconeogenesis by extracellular NAD^+ (Martins et al., 2006). The experiments shown in Figure 3 were done with the purpose of verifying the response to NAD^+ of the liver from Holtzman control and arthritic rats in retrograde perfusion. The responses of oxygen uptake and gluconeogenesis to lactate infusion in retrograde perfusion of both livers from control and arthritic rats were similar to the responses in antegrade perfusion (compare Figures 2 and 3). Pyruvate production, on the other hand, was higher in the arthritic condition in retrograde perfusion. The infusion of NAD^+ produced transient increases in oxygen uptake and gluconeogenesis in both the control and arthritis conditions without the initial inhibitory phase. The increments were clearly smaller in the arthritis condition. In the control condition the peak increment in oxygen uptake was equal to 0.389 ± 0.007 μmol min^{-1} g^{-1} compared to only 0.175 ± 0.028 μmol min^{-1} g^{-1} in the arthritis condition ($p < 0.001$). For glucose production the corresponding peak increments were 0.177 ± 0.019 and 0.026 ± 0.011 μmol min^{-1} g^{-1}, respectively ($p < 0.001$). In contrast to glucose production and oxygen uptake, pyruvate production was transiently decreased in retrograde perfusion, similarly to what happened in antegrade perfusion (see Figures 2 and 3).

Previous studies have demonstrated the Ca^{2+}-dependence of most effects of extracellular NAD^+ (Broetto-Biazon et al., 2004; Martins et al., 2006). Figure 4 illustrates the attempts of testing this Ca^{2+}-dependence in livers from arthritic rats. Calcium-free perfusion had little influence on the response to lactate. Upon NAD^+ infusion, the initial inhibitions of glucose production and oxygen uptake were no longer seen in the absence of Ca^{2+} and the initial inhibition of pyruvate production was drastically diminished. Only small stimulatory effects on oxygen uptake and glucose were still visible in the control condition, but pratically absent in the arthritis condition.

Indomethacin, an inhibitor of prostaglandin, thromboxane, and prostacyclin synthesis (Vane, 1971), diminishes the action of extracellular NAD^+ on oxygen uptake and gluconeogenesis. (Martins et al., 2006). The results shown in Figure 5 are from experiments whose purpose was to investigate the sensitivity of livers from arthritic rats to indomethacin. The compound (30 μM) was introduced at 30 minutes perfusion time and it produced a small diminution of glucose production before NAD^+ infusion without affecting pyruvate production and oxygen uptake. Indomethacin completely eliminated the inhibitory actions of NAD^+ on glucose production and oxygen uptake in both control and arthritis conditions and the inhibition of pyruvate production was considerably reduced (compare Figures 2 and 5). Stimulation of glucose production and oxygen uptake in the control condition was also considerably reduced by indomethacin.

Bromophenacyl bromide is an inhibitor of phospholipase A2 (Volwerk et al., 1974) and it also produces substantial changes in the action of extracellular NAD^+. In the present work bromophenacyl bromide was infused at a concentration of 100 μM. As revealed by Figure 6, the compound caused a slight declining tendency to glucose production. The action of NAD^+ was changed in that the initial inhibitions of oxygen uptake and gluconeogenesis were no longer present and replaced by transient stimulations. The peak oxygen uptake stimulations were 0.363±0.058 and 0.289±0.049 μmol min^{-1} g^{-1}, respectively, for the control and arthritis condition (p = 0.38). For gluconeogenesis the corresponding peak stimulations were 0.376±0.109 and 0.068±0.008 μmol min^{-1} g^{-1} (p = 0.048). Pyruvate production, on the other hand, was still inhibited by NAD^+ in the presence of bromophenacyl bromide, but to a lesser extent when compared with the inhibition in the absence of bromophenacyl bromide (compare with Figure 2).

Nordihydroguaiaretic acid is an inhibitor of leukotriene synthesis (Chang et al., 1984). As shown in Figure 7, when infused at a concentration of 25 μM, it did not produce significant changes in the response to lactate infusion, but it modified the action of NAD^+ in both control and arthritis conditions. In the control condition, the stimulatory phenomenon which occurred shortly after the initial inhibition (see Figure 2) was abolished. The peak decreases in glucose production and oxygen uptake were not modified, 0.269±0.068 and 0.593±0.168 μmol min^{-1} g^{-1}, respectively (see Table 2). There was some tendency of shifting the peak times, however, which were equal to 0.87±0.08 and 1.27±0.08 minutes for glucose production and oxygen uptake, respectively (compare with data in Table 2). In the

arthritis condition, the extent of the peak decrease in glucose production was equal to 0.373±0.028 μmol min^{-1} g^{-1}, significantly greater than that in the absence of nordihydroguaiaretic acid (see Table 2). The peak decrease in oxygen uptake was 0.992±0.069 μmol min^{-1} g^{-1}, similar to that one found in the absence of nordihydroguaiaretic acid (see Table 2). The recovery in oxygen uptake was incomplete, however, in that it remained clearly below the values found in the presence of lactate alone during the remaining time of NAD^+ infusion.

Discussion

The hepatic response of Wistar and Holtzman rats to NAD^+

Before discussing the modifications in the response to extracellular NAD^+ caused by the adjuvant induced arthritis in Holtzman rats, it seems appropriate to analyze the differences in the hepatic response to this agonist between Holtzman and Wistar rats. The hepatic response of gluconeogenesis and the associated oxygen uptake to extracellular NAD^+ of Wistar rats has been recently investigated using the same experimental system so that comparisons are straightforward (Martins et al., 2006). A good correlation between the changes in oxygen uptake and gluconeogenesis has been found and experiments with respiratory inhibitors have suggested that inhibition and possibly also stimulation of oxygen uptake are the primary events influencing gluconeogenesis by virtue of the energy requirements of this biosynthetic route (Martins et al., 2006). The gluconeogenic capacity of the liver from Wistar and Holtzman rats is practically the same (Martins et al., 2006). Similar is also the diminution of hepatic gluconeogenesis from lactate by the arthritis disease in Wistar (−43%; Fedatto-Jr et al., 1999) and Holtzman rats (−49%). The responses to NAD^+ of both strains are also similar in that the initial inhibition is followed by stimulation of both oxygen uptake and gluconeogenesis and that these actions are Ca^{2+}-dependent (Martins et al., 2006). Wistar rats also present transient increases in portal pressure (Gimenes et al., 2006). However, the kinetics and the extent of gluconeogenesis and oxygen inhibition or stimulation are different in both strains. The initial inhibitory phase in the Holtzman strain is much shorter and smaller. This can be quantified by the Holtzman/Wistar ratios of the initial inhibitions of glucose production and oxygen uptake by 100 μM NAD^+ which are 0.42 and 0.45, respectively, as can be calculated from the data in Table 2 and those ones presented by Martins et al. (2006). The opposite occurs with the stimulation that follows inhibition, the Holtzman/Wistar ratios in this case being equal to 1.97 and 2.28, respectively, for gluconeogenesis and oxygen uptake. One is tempted to conclude that the inhibitory signal of NAD^+ in Holtzman rats is less effective whereas the opposite occurs with the stimulatory signal. However, it is appropriate to note that in retrograde perfusion the inhibitory signal is absent in both Wistar (Martins et al., 2006) and Holtzman rats. This occurs, most probably, because in both strains the messengers for inhibition are generated in the

presinuoidal region, so that they do not reach the hepatocyte in retrograde perfusion (Gimenes et al., 2006). A comparison of the stimulatory effect in retrograde perfusion would, thus, tell us if this signal is in fact more intense in Holtzman rats. Calculation of the Holtzman/Wistar ratio for gluconeogenesis and for the oxygen uptake increment in retrograde perfusion caused by 100 μM NAD^+ produces values of 0.84 and 0.97, respectively (Martins et al. 2006). It is thus likely that the strength of the stimulatory signal of NAD^+ is actually similar in both Wistar and Holtzman rats, the increased stimulation in antegrade perfusion in the latter being most probably the consequence of a shorter and less pronounced inhibitory action. Inhibition of eicosanoid production by indomethacin (Vane, 1971) practically abolished the initial inhibition, a phenomenon common to Wistar (Martins et al. 2006) and Holtzman rats. This observation strongly suggests that the initial inhibition is caused by prostanoids derived from Kupffer cells, which exert similar effects when given to the perfused liver (Imesh and Rous, 1975; Altin and Bygrave, 1988; Häussinger et al 1988). In this sense the fact that the inhibitory phase is less pronounced and less persistent in the liver of Holtzman rats corroborates some previous observations about different responses of Wistar and Holtzman rats to agents whose action depends on eicosanoid synthesis. It has been reported, for example, that bradykinin induces dose-dependent contraction of relaxed mesenteric arterial rings from Holtzman rats, but not from Wistar rats, an action which is mediated by eicosanoids (Weinberg et al., 1997). Experiments on platelet aggregation induced by arachidonic acid, a precursor of eicosanoids, can also be regarded as an indicator that Holtzman rats may respond differently when eicosanoids are involved. Platelets from the Holtzman strain were more sensitive to arachidonic acid than were those from other strains (Dwyer and Meyers, 1986). Participation of arachidonic acid, as a precursor of eicosanoids, in the effects of NAD^+ is also suggested by the action of nordihydroguaiaretic acid, which tended to enhance the persistence of the inhibitory effects. Since this compound inhibits the route leading from arachidonic acid to leukotriene synthesis (Chang et al., 1984), it is reasonable to expect an increased flux through the route leading to prostanoid synthesis due to the increased availability of this fatty acid for the cyclooxygenase reaction. This interpretation is corroborated by the observation that exposure of perfused livers to arachidonic acid, stimulates hepatic vasoconstriction caused by eicosanoids (Dieter et al., 1987).

The hepatic response of arthritic Holtzman rats to NAD^+

The results of the present work reveal that adjuvant induced arthritis is able to modify the response of the liver from Holtzman rats to extracellular NAD^+. Arthritis modifies the response to extracellular NAD^+ by increasing (in relative terms) and prolonging the inhibitory phase and diminishing stimulation of both gluconeogenesis and oxygen uptake. The diminished stimulation of both gluconeogenesis and oxygen uptake could be simply reflecting the diminished hepatic gluconeogenic capactity of the arthritic condition (Fedatto-Jr et al., 1999). The stronger and more persistent inhibitions of gluconeogenesis and oxygen uptake and the more accentuated increase in perfusion pressure caused by NAD^+ in the liver of arthritic rats, however, are most likely the consequence of an enhanced participation of prostanoids. The sensitivity of the initial inhibitions caused by NAD^+ to indomethacin and bromophenacyl bromide (Vane, 1971; Volwerk et al., 1974; Hatch et al., 1993) makes it highly probable that they are caused by products from the cyclooxygenase pathway and there is wide consensus that the capacity of eicosanoid synthesis in non-parenchymal liver cells is increased during inflammation (Pérez et al., 2006). In liver macrophages during inflammation a signal pathway triggered by bound endotoxin involves a mitogen-activated protein kinase and leads to the activation of phospholipase A2 and the synthesis of eicosanoids (Decker, 1998). Liver biopsies of patients with juvenile chronic polyarthritis have also revealed nonspecific infiltrations of portal fields with lymphocytes (Rau, 1977), a probable source of arachidonic acid for eicosanoid production (Goldyne and Stobo, 1982). In rats with adjuvant-induced arthritis, prostaglandins (especially PGE1) have been proposed to be the main cause for the diminished rates of hepatic drug transformation detected in experiments with the isolated perfused liver (Ishizuki et al., 1986). It should be remarked, however, that although the higher production rates of eicosanoids could be responsible for the apparently accentuated influence of these agents in the liver of arthritic animals, changes in the response of the target cells, mainly hepatocytes, are equally possible.

Concerning the more accentuated increase in perfusion pressure upon NAD^+ infusion in livers from arthritic rats it is worth to mention that prostaglandin E2 (PGE2) release by macrophages has been demonstrated to inhibit NO production in hepatocytes (Griffon et al., 1998). A reduced production of this vasodilator induced by eicosanoids could, thus, be an important cause for the increased portal perfusion

pressure of livers from arthritic rats and also for the more pronounced increment caused by NAD^+.

Although it seems clear that the transient inhibition of oxygen uptake and gluconeogenesis by extracellular NAD^+ are the consequence of a stimulation of the release of products derived from the cyclooxygenase pathway, the bulk of the effects is certainly the result of highly complex interactions and modifications. As suggested previously (Martins et al., 2006), the stimulation that follows inhibition could be independent of eicosanoid production because it was still observed in the presence of bromophenacyl bromide, which by inhibiting phospholipase A2 in Kupffer cells also abolishes the production of these agents (Volwerk et al., 1974; Hatch et al., 1993). However, bromophenacyl bromide is not specific. It has been shown, for example, to inhibit phospholipase C and alcohol dehydrogenase and to modify sulfhydryl groups (Kyger and Franson, 1984). It cannot be excluded, thus, that the transient stimulation caused by extracellular NAD^+ in the presence of bromophenacyl bromide represents a different phenomenon generated by the action of this inhibitor which is not significantly operative in its absence.

Absence of correlation between the oxygen uptake and pyruvate production changes caused by NAD^+

The almost strict correlation between the changes in oxygen uptake and gluconeogenesis caused by NAD^+ reported previously (Martins et al., 2006) were confirmed in the present work. Surprisingly, no such correlation was found between the oxygen uptake changes and pyruvate production. This observation disproves a previous notion that the diminution of pyruvate release caused by extracellular NAD^+, which also occurs when glycolysis from endogenous sources in operative in the liver, is the consequence solely of oxygen uptake inhibition (Broetto-Biazon et al., 2004). Inhibition of oxygen uptake generally leads to a diminution in the pyruvate concentration and release because the $NADH/NAD^+$ ratio is increased, a phenomenon that immediately reflects in the lactate dehydrogenase equilibrium (Sies, 1982). There are many mechanisms that could be responsible for this phenomenon. It could be, for example, that the NAD^+ signal inhibits the activity of the malate-aspartate shuttle, diminishing the oxidation of cytosolic reducing equivalents (Shiota et al., 1994). This would increase the cytosolic $NADH/NAD^+$ irrespective of the actual change in the mitochondrial electron flow, inhibition or stimulation.

Concluding remarks

In conclusion, the results of the present work reinforce the hypothesis that the paracrine action of NAD^+ is at least partly mediated by eicosanoids and that this action can be modified by diseases involving these agents. There are still many aspects that must be clarified about the action of NAD^+ in the liver, including the receptors that are involved, the main molecular eicosanoid species and also the secondary agents responsible for gluconeogenesis and oxygen uptake stimulation. Finally, the relatively small differences in the action of NAD^+ on the hepatic metabolism of two strains of the same species (Wistar and Holtzman rats) leads one to expect even greater differences between different species.

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Table 1

Parameters related to the portal pressure changes caused by 100 μM extracellular NAD^+. The parameters were obtained from the experiments illustrated by Figure 1. The peak pressure increase was calculated by subtracting the basal pressure from the maximal pressure after initiation of NAD^+ infusion. The time for half-maximal decay was obtained by numerical interpolation (Stineman's interpolation) and subtracted from the corresponding peak time. The *p* values were obtained by applying Student's t test.

Condition	Peak pressure increase (mm Hg)	Peak time (minutes)	Time for half-maximal decay, $t_{½}$ (minutes)
Control (n=3)	3.15±0.28	1.14±0.03	1.18±0.07
Arthritic (n=3)	4.40±0.18*	1.25±0.05‡	1.47±0.15†

*p = 0.02; $^{‡}p$ = 0.13; $^{†}p$ = 0.16.

Table 2

Peak times, peak decreases and times for half-maximal recovery in glucose production, oxygen uptake and pyruvate production during NAD^+ infusion in livers from control and arthritic rats. The data were obtained from the experimental results shown in Figure 2. The peak decreases were calculated from the rates of glucose production and oxygen uptake after 34 minutes lactate infusion (F_L) and the corresponding minimal rates (F_{-peak}) during NAD^+ infusion, i.e., peak decrease = $F_{-peak} - F_L$.

Metabolic variable	Peak decrease (F_{-peak}) (μmol min^{-1} g^{-1})		Peak time (minutes)		Time for half-maximal recovery, $t_{½}$ (minutes)	
	Control (n=5)	Arthritic (n=6)	Control (n=5)	Arthritic (n=6)	Control (n=5)	Arthritic (n=6)
Glucose production	0.291±0.042[a] (34.2±2.7%)[j]	0.224±0.031[a] (44.0±2.9%)[j]	0.67±0.05[b]	1.22±0.20[b]	0.62±0.08[c]	2.82±0.49[c]
Oxygen uptake	0.631±0.158[d] (19.7±4.3%)[k]	0.984±0.125[d] (36.0±4.4%)[k]	1.05±0.03[e]	1.89±0.17[e]	0.83±0.15[f]	2.95±0.60[f]
Pyruvate production	0.381±0.025[g] (66.8±2.4%)[λ]	0.346±0.039[g] (66.9±4.9%)[λ]	1.07±0.04[h]	1.25±0.04[h]	1.18±0.03[i]	1.54±0.12[i]

[a]$p = 0.22$; [b]$p = 0.037$; [c]$p = 0.003$; [d]$p = 0.11$; [e]$p = 0.002$; [f]$p = 0.012$; [g]$p = 0.49$; [h]$p = 0.012$; [i]$p = 0.027$; [j]$p = 0.038$; [k]$p = 0.028$; [λ]$p = 0.99$.

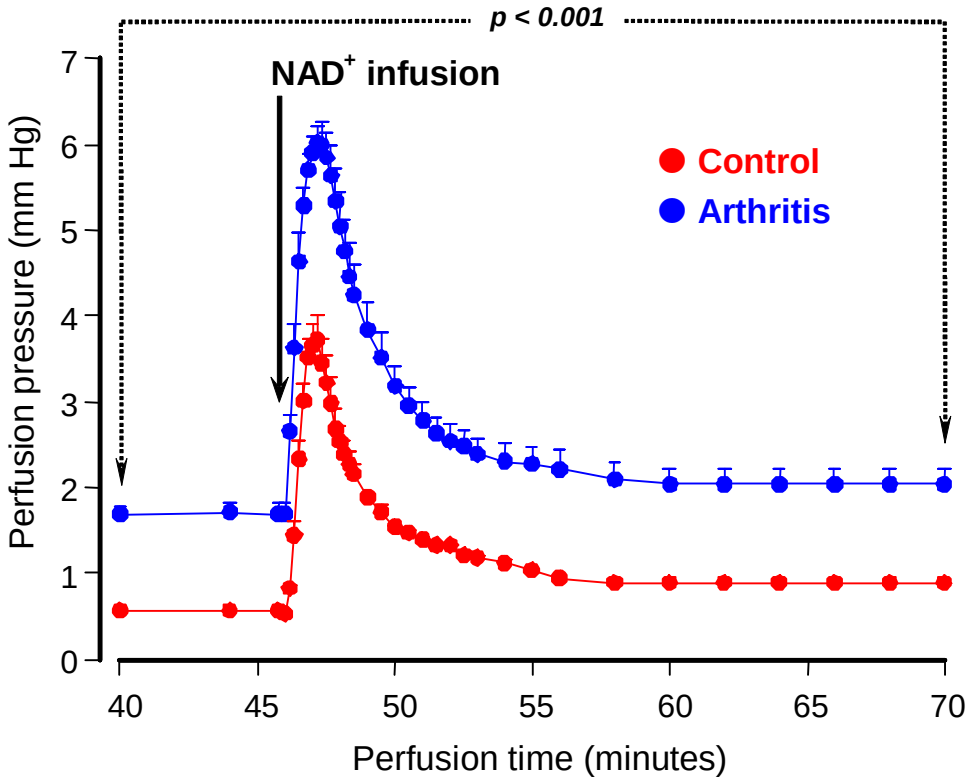

Figure 1. ***Changes in portal perfusion pressure as a function of time in the perfused rat liver in consequence of portal NAD$^+$ infusion.*** Livers of fasted rats were perfused in the antegrade mode as described in Materials and methods. The portal perfusion pressure was monitored by means of a pressure transducer. NAD$^+$ was infused into the portal vein at a concentration of 100 μM. The data points are means ± mean standard errors of 3 liver perfusion experiments. Statistical significance of the differences between the control (●) and the arthritic condition (●) was evaluated by means of two-way variance analysis with post-hoc Duncan's multiple range testing. The time span presenting significant differences between the control and tumor-bearing conditions is indicated.

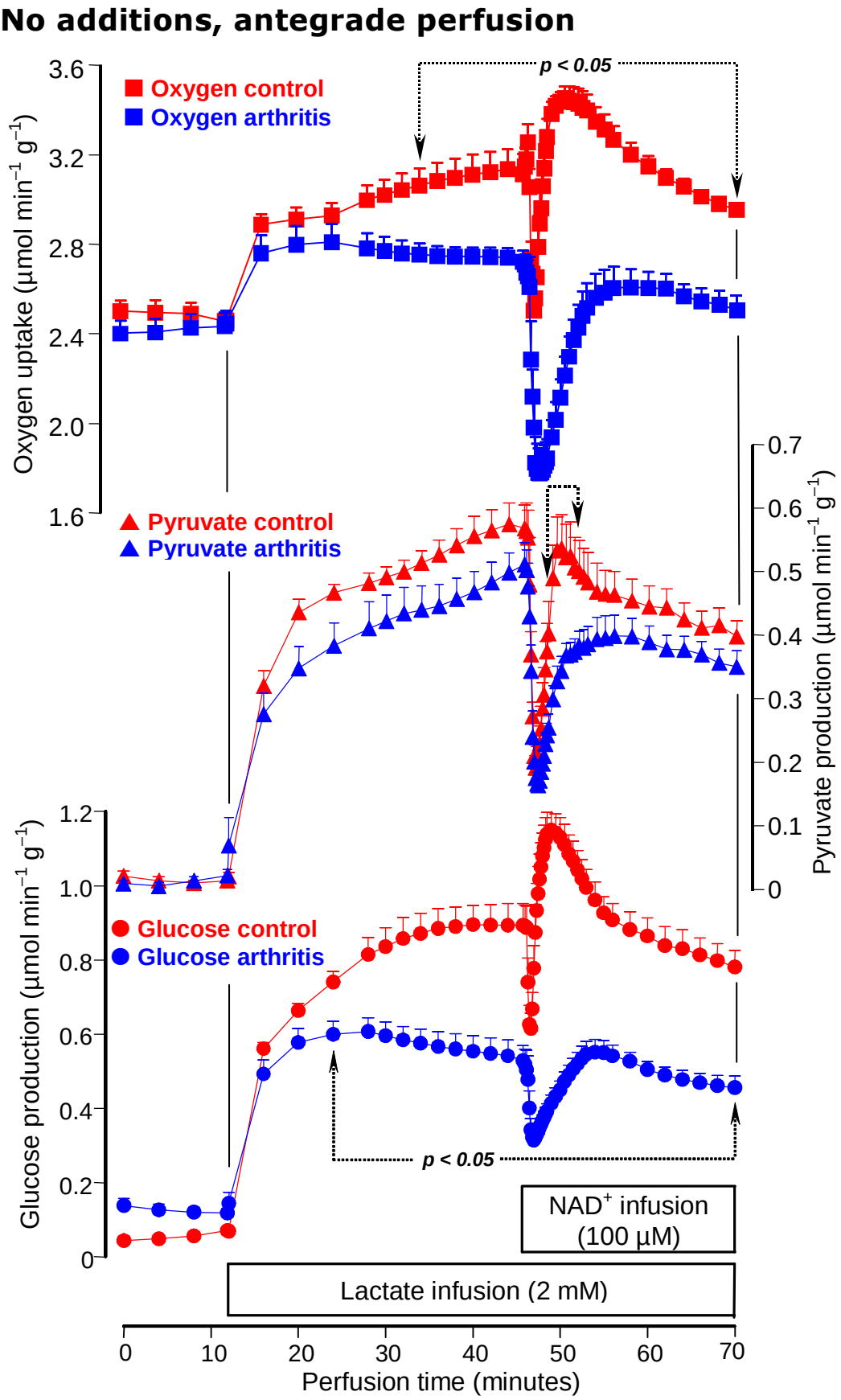

Figure 2. **Influence of arthritis on the actions of NAD$^+$ on glucose and pyruvate productions and oxygen uptake in the rat liver during antegrade perfusion.** Livers from fasted rats were perfused. Lactate (2 mM) and NAD$^+$ (100 µM) were infused as indicated. The data are the means ± mean standard errors of 5 (control) and 6 (arthritic) liver perfusion experiments. The time spans presenting significant differences between the control and arthritic conditions are indicated (two-way variance analysis, with post hoc Duncan's multiple range testing).

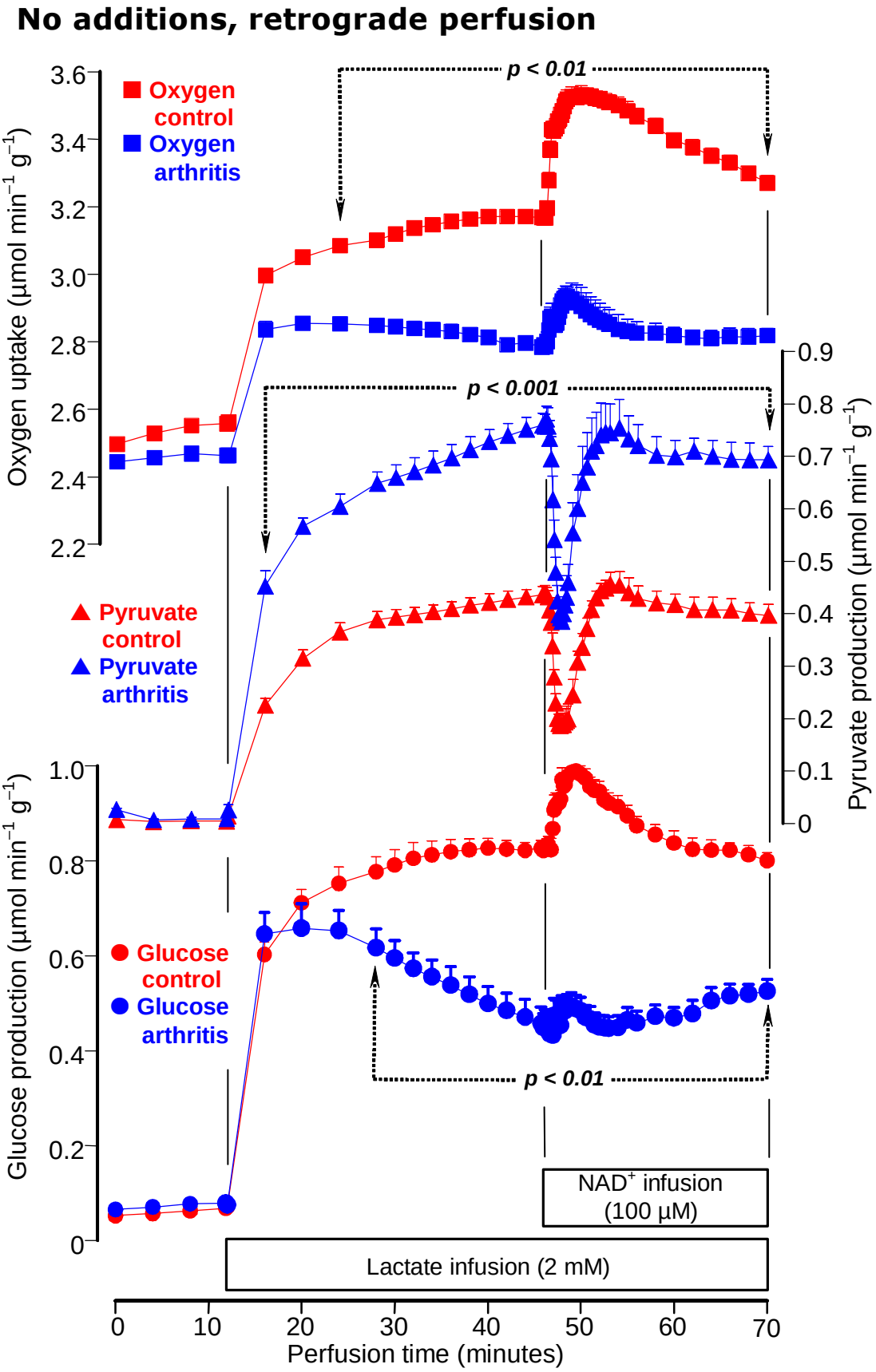

Figure 3. **Influence of arthritis on the actions of NAD^+ on glucose and pyruvate productions and oxygen uptake in the rat liver during retrograde perfusion.** Livers from fasted rats were perfused. Lactate (2 mM) and NAD^+ (100 µM) were infused as indicated. The data represent the means ± mean standard errors of 4 liver perfusion experiments for each condition. The time spans presenting significant differences between the control and arthritic conditions are indicated (two-way variance analysis, with post hoc Duncan's multiple range testing).

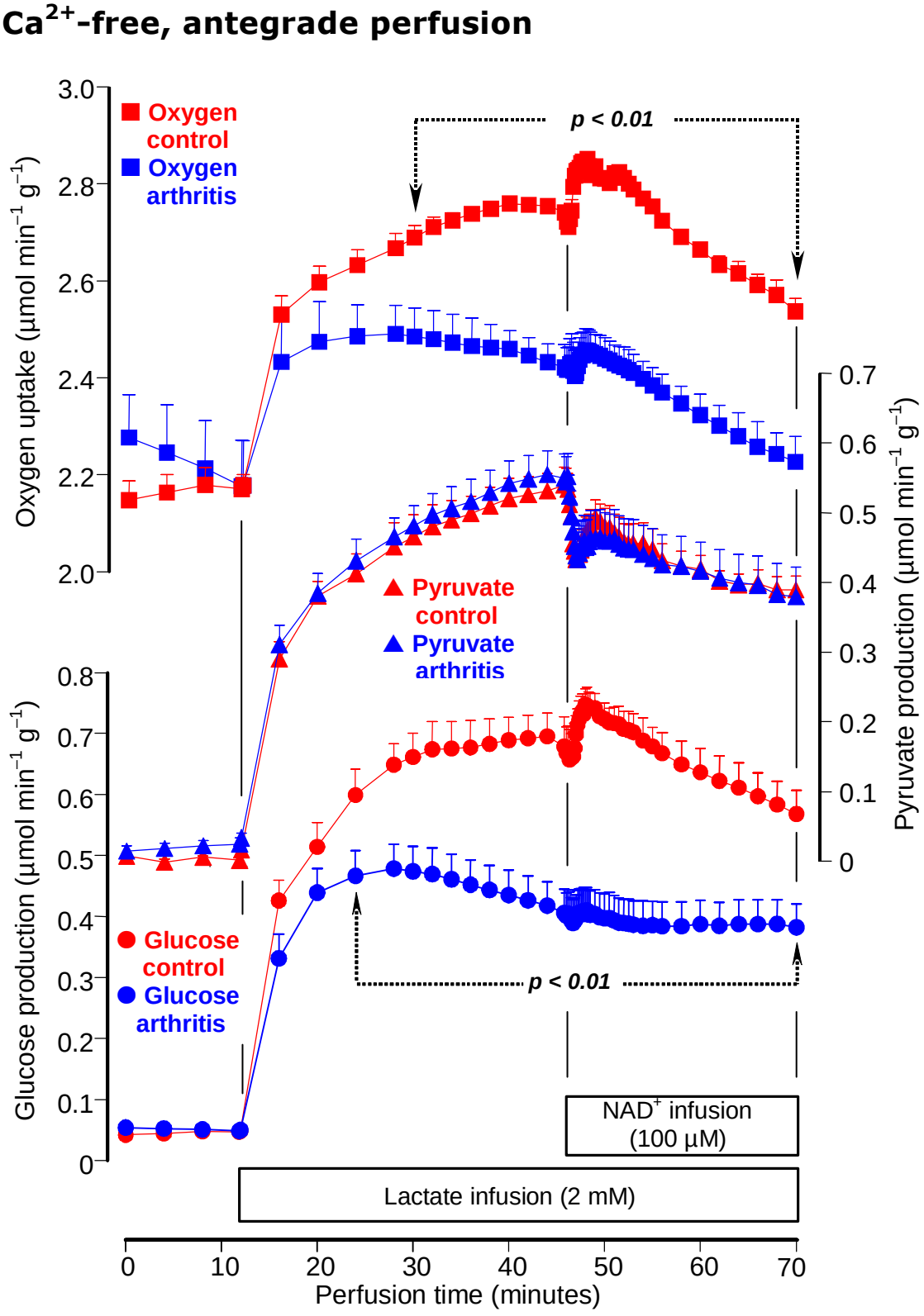

Figure 4. ***Influence of Ca^{2+}-free perfusion on the actions of NAD^+ on glucose and pyruvate productions and oxygen uptake in the rat liver during retrograde perfusion.*** Livers from fasted rats were perfused with Ca^{2+}-free medium after depletion of the cellular calcium stores. Lactate (2 mM) and NAD^+ (100 µM) were infused as indicated. The data represent the means ± mean standard errors of 4 (control) and 6 (arthritic) liver perfusion experiments. The time spans presenting significant differences between the control and arthritic conditions are indicated (two-way variance analysis, with post hoc Duncan's multiple range testing).

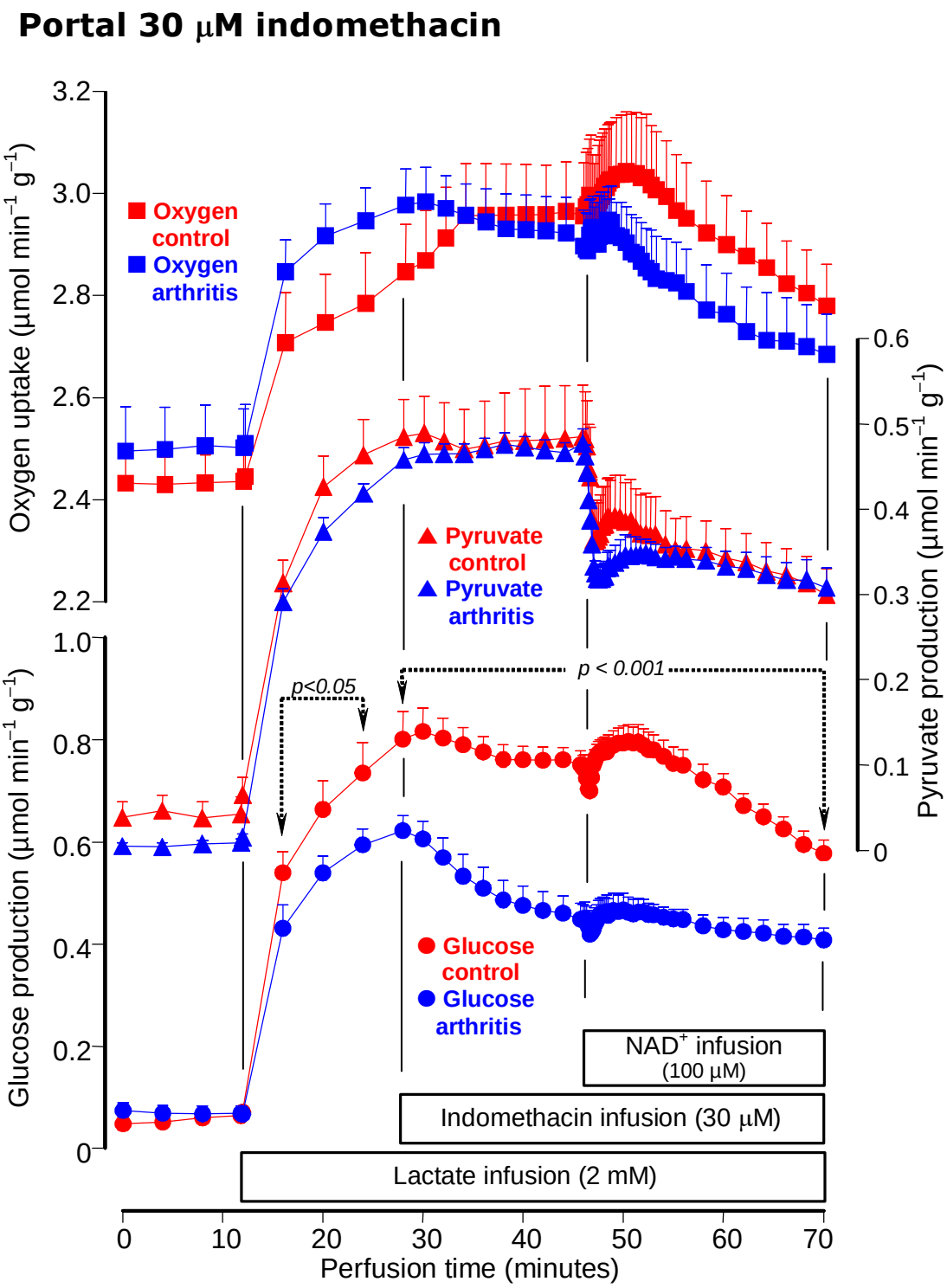

Figure 5. ***Influence of indomethacin on the actions of NAD$^+$ on glucose and pyruvate productions and oxygen uptake in the rat livers of control and arthritic rats.*** Livers from fasted rats were perfused in the antegrade mode with 30 μM indomethacin. Lactate (2 mM) and NAD$^+$ (100 μM) were infused as indicated. The data represent the means ± mean standard errors of 6 liver perfusion experiments for each condition. The time spans presenting significant differences between the control and arthritic conditions are indicated (two-way variance analysis, with post hoc Duncan's multiple range testing).

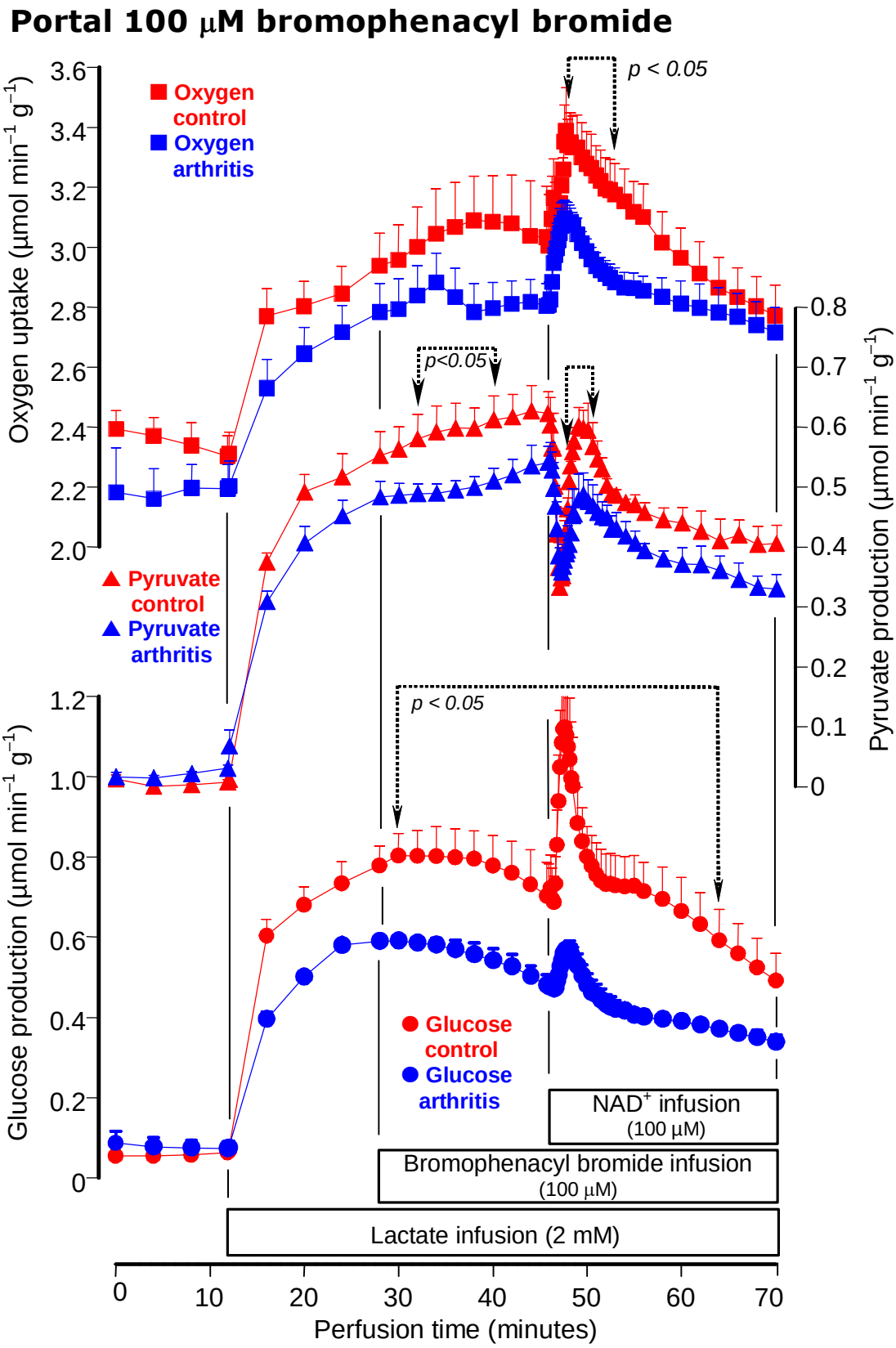

Figure 6. ***Influence of bromophenacyl bromide on the actions of NAD$^+$ on glucose and pyruvate productions and oxygen uptake in the rat livers of control and arthritic rats.*** Livers from fasted rats were perfused in the antegrade mode with 100 μM bromophenacyl bromide. Lactate (2 mM) and NAD$^+$ (100 μM) were infused as indicated. The data represent the means ± mean standard errors of 3 liver perfusion experiments for each condition. The time spans presenting significant differences between the control and arthritic conditions are indicated (two-way variance analysis, with post hoc Fisher's least significant difference testing).

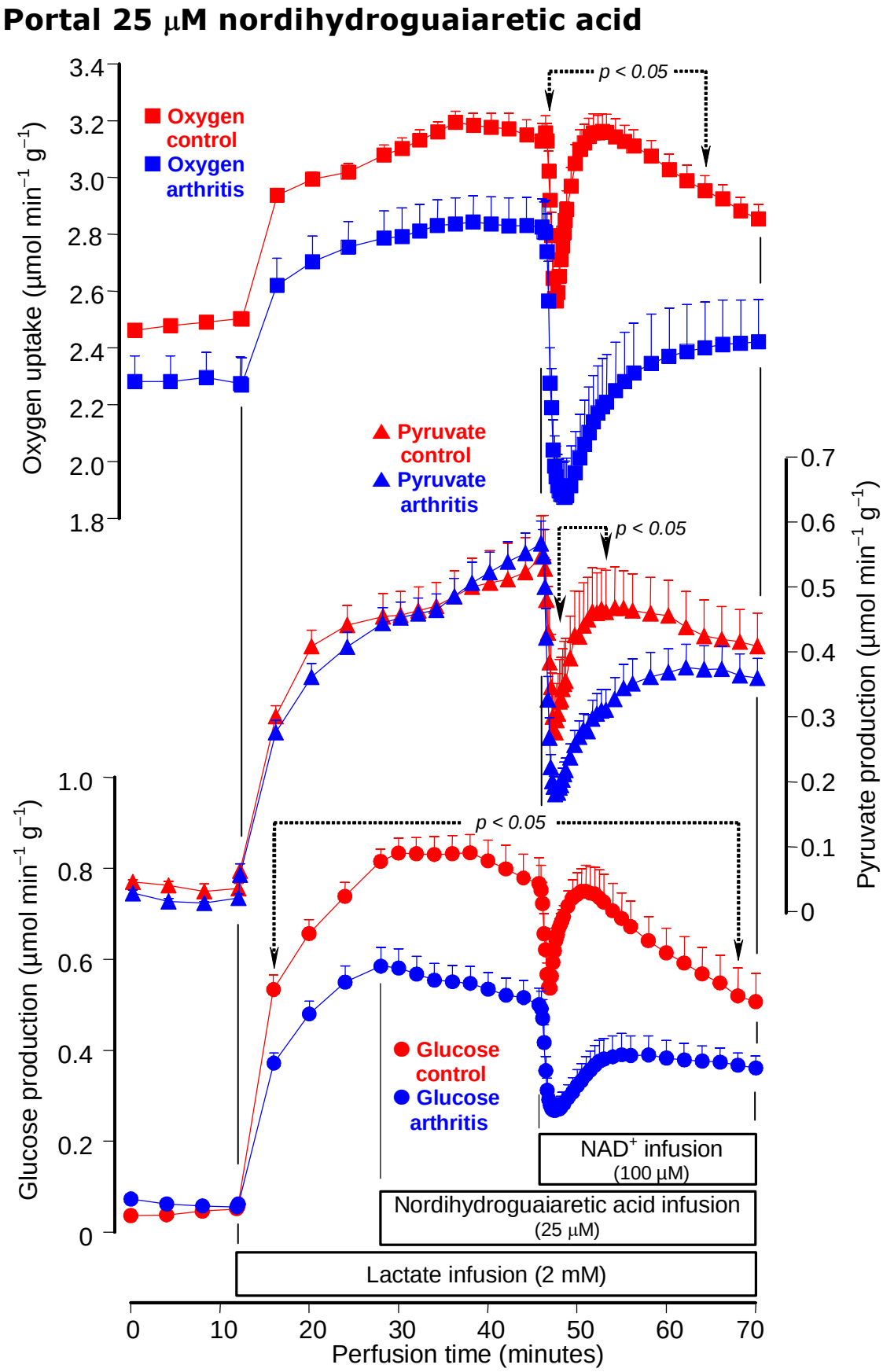

Figure 7. ***Influence of nordihydroguaiaretic acid on the actions of NAD⁺ on glucose and pyruvate productions and oxygen uptake in the rat livers of control and arthritic rats.*** Livers from fasted rats were perfused in the antegrade mode with 25 μM nordihydroguaiaretic acid. Lactate (2 mM) and NAD^+ (100 μM) were infused as indicated. The data are the means ± mean standard errors of 5 (control) and 10 (arthritic) liver perfusion experiments. The time spans presenting significant differences between the control and arthritic conditions are indicated (two-way variance analysis, with post hoc Duncan's multiple range testing).

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