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The action of extracellular NAD^+ in the liver of healthy and tumor-bearing rats: Model analysis of the tumor-induced modified response

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ABSTRACT

The chronic inflammatory state induced by cancer is expected to affect the actions of extracellular NAD^+ in the liver because these are largely mediated by eicosanoids. Under this assumption the present work was planned to investigate the influence of the Walker-256 tumor on the action of extracellular NAD^+ on metabolism and hemodynamics in the perfused rat liver. The experiments were done with livers from healthy and tumor-bearing rats with measurements of gluconeogenesis from lactate, pyruvate production, oxygen consumption and portal pressure. A model describing the biphasic effects of NAD^+ was proposed as an auxiliary worktool for interpretation. The Walker-256 tumor modified the responses of metabolism to extracellular NAD^+ by delaying the peak of maximal responses and by prolonging the inhibitory effects. The transient increase in portal perfusion pressure caused by NAD^+ was enhanced and delayed. The model was constructed assuming the mediation of a down-regulator (inhibition), an up-regulator (stimulation) and receptor dessensitization. Analysis suggested that the productions of both the down- and up-regulators were substantially increased and delayed in time in the tumor-bearing condition. Since the regulators are probably eicosanoids, this analysis is consistent with the increased capacity of producing these agents in the chronic inflammatory state induced by cancer.

Introduction

The alterations in the profile of hormones and cytokines during cancer cachexia induce the establishment of a chronic inflammatory state 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 syndrome, are partly mediated by eicosanoids (Lundholm et al., 1994, 2004). Prostaglandin E2 (PGE2), for example, is produced in great quantities by both the tumor and the host cells (Siddiqui and Williams, 1989). 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). In addition to the production of eicosanoids (Siddiqui and Williams, 1989) the chronic inflammatory picture induced by cancer cachexia could also be affecting the expression of the various types of eicosanoid receptors.

For all these reasons it seems reasonable to expect a modified response of the liver of cachectic 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., 2003), 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 catalyzed 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. Most of these effects are Ca^{2+}-dependent and also 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 three different 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-

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ribose, free nicotinamide, nucleotide nicotinic acid-adenine dinucleotide phosphate (NAADP) and cyclic ADP-ribose(cADPR) (Lee, 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 strengthens 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 cachexia and its accompanying chronic inflammatory state on the action of extracellular NAD^+ on some metabolic and functional parameters in the isolated perfused rat liver. Rats bearing the Walker-256 tumor were chosen as a model for cachexia (Guaitani et al., 1982). Using this model several metabolic parameters have already been investigated in the isolated perfused liver, as for example the urea cycle and gluconeogenesis (Corbello-Pereira et al., 2004). 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). As a worktool for interpretation a mathematical model is being proposed for the action of extracellular NAD^+. The results should allow the first insight into the influence of cancer 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).

Animals and tumor implantation

Male Wistar rats weighing 200–250 g and fed with a standard laboratory diet (Nuvilab®) were used in all experiments. The Walker-256 carcinosarcoma, an established model for the study of cancer cachexia, was implanted into the rats as described previously (Vicentino et al., 2002a; Corbello-Pereira et al., 2004). Walker-256 carcinosarcoma cells were injected sub-cutaneously (2×10^7 cells per rat), after assessment of their viability with the method of trypan blue exclusion in a Neubauer chamber. The Walker-256 carcinoma cells were suspended in phosphate-buffered saline at pH 7.4 (137 mM NaCl, 2.7 mM KCl and 16.5 mM phosphate). The tumor cells were injected into the right flank of the rats (tumor-bearing rats). The control rats were injected with phosphate-buffered saline. The perfusion experiments were done after 14 days. The cachectic conditions of the Walker-256 tumor-bearing rats utilized in the present study have been described in a previous study (Vicentino et al., 2002a). All experiments were done in accordance with the internationally accepted recommendations in the care and use of animals.

Liver perfusion

Male albino rats (Wistar), weighing 200–250 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 provided 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 done. In some selected experiments, however, perfusion was switched to the retrograde mode (hepatic vein→portal vein). 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 $CaCl_2$.

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 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.

Modelling the liver response to extracellular NAD^+

The responses of the liver to extracellular NAD^+ in the control and tumor-bearing states were also analyzed in terms of a mathematical model in order to get some insight into the possible reasons for the tumor-bearing dependent modifications. Previous work has shown that the effects of NAD^+ are a combination of inhibitory and stimulatory effects (Broetto-Biazon et al., 2004; Gimenes et al., 2006; Martins et al., 2006). Under gluconeogenic conditions, particularly, inhibition of oxygen uptake and gluconeogenesis is followed by a recovery and, depending on the NAD^+ concentration, by transient stimulation. The latter is more accentuated at low than at high NAD^+ concentrations. Furthermore, in retrograde perfusion, inhibition is practically absent, stimulation being the sole effect, an observation that strongly indicates that the recovery from inhibition must also include a stimulatory component (Martins et al., 2006). With these facts in mind it can be assumed, for the sake of simplicity, that the modifications caused by NAD^+ depend on the combined action of at least two effectors, a down-regulator and an up-regulator. The up-regulator stimulates metabolism, whereas the down-regulator diminishes the sensitive metabolic fluxes. Using the formulations for stimulation (Gall et al., 2000) and inhibition (Schulz, 1994) that are traditionally applied to enzymatic systems, the following equation can be used to describe the behavior of the metabolic flux, $F(t)$, following the onset of NAD^+ infusion:

$$F(t) = F_{\text{basal}}\left(1+\frac{\lambda_{\max}C_{\text{up}}(t)}{K_{\text{up}}+C_{\text{up}}(t)}\right)\left(\frac{1}{1+C_{\text{down}}(t)/K_{\text{down}}}\right). \quad (1)$$

In Eq. (1), F_{basal} represents the basal metabolic flux (the steady-state rate before NAD^+ infusion), $C_{\text{up}}(t)$ the time-dependent concentration of the up-regulator, K_{up} the stimulation constant, $\lambda_{\max}$ the maximal stimulation, $C_{\text{down}}(t)$ the time-dependent concentration of the down-regulator and K_{down} the inhibition constant. If no up-regulator is present, i.e., if $C_{\text{up}}(t)=0$, inhibition will be the only effect; and, if no down-regulator is present, i.e., $C_{\text{down}}(t)=0$, stimulation is the sole effect. If stimulation is far from saturation, i.e., if $K_{\text{up}} \gg C_{\text{up}}(t)$, the second multiplicative term in Eq. (1) reduces to $[1+(\lambda_{\max}/K_{\text{up}})C_{\text{up}}(t)]$. In this case only the ratio $\lambda_{\max}/K_{\text{up}}$ can be determined.

An essential feature of the formulations given above is that both C_{down} and C_{up} are functions of time after initiation of NAD^+ infusion. This can be adequately described by a set of differential equations. The rate of change of the down-regulator concentration with respect to time ($dC_{\text{down}}(t)/dt$) is here proposed to depend on the rate of its production induced by NAD^+, v_{down}, and on the rate of its decomposition. Decomposition is catalyzed by an enzymic system obeying classical Michaelis–Menten kinetics. Eq. (2) incorporates these and other assumptions:

$$\frac{dC_{\text{down}}(t)}{dt} = \frac{v_{\text{down}}\left(1-e^{-\beta t}\right)}{1+C_{\text{des}}/K_{\text{des1}}} - \frac{V_{\text{downd}}C_{\text{down}}}{K_{\text{downd}}+C_{\text{down}}}. \quad (2)$$

V_{downd} is the maximal rate of the down-regulator decomposition and K_{downd} the corresponding Michaelis–Menten constant. The maximal rate of the down-regulator

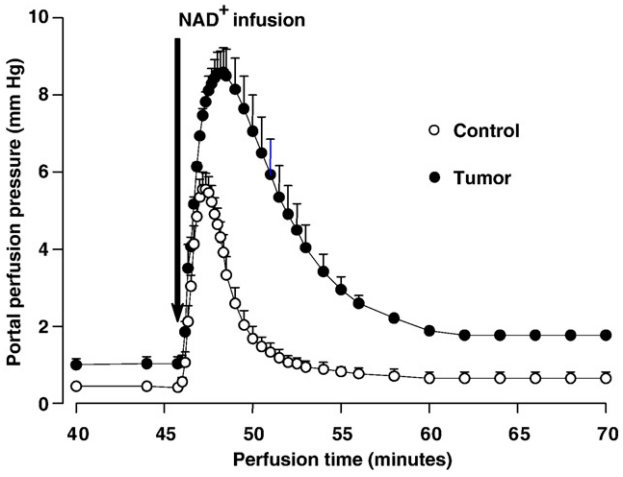

Fig. 1. Changes in portal perfusion pressure as a function of time in the perfused rat liver in consequence of arterial NAD^+ infusion. Livers of fasted rats were perfused in the antegrade mode as described in Materials and methods. Portal perfusion pressures were monitored simultaneously by means of 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.