The Influence of Ca\(^{2+}\) on Gluconeogenesis Stimulation by Glucagon in the Liver of Arthritic Rats

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ABSTRACT

Ca\(^{2+}\) participates in the stimulation of hepatic gluconeogenesis by glucagon and there is evidence that Ca\(^{2+}\) fluxes are modified in arthritic rats. These findings raise the question whether hepatic gluconeogenesis in arthritic rats responds differently to glucagon and Ca\(^{2+}\). The experimental system was the isolated perfused rat liver. In the presence of Ca\(^{2+}\), stimulation of hepatic gluconeogenesis by glucagon in arthritic rats was equal to that in normal rats in absolute terms, but higher in relative terms (104.5 and 45.2%, respectively). In the absence of Ca\(^{2+}\), however, stimulation of hepatic gluconeogenesis by glucagon in arthritic rats was smaller in both absolute and relative terms (18.5 and 41.9%, respectively). It can be concluded that the Ca\(^{2+}\)-dependent component of gluconeogenesis activation by glucagon is more important in arthritic than in normal rats.

Key words: Arthritis, liver, gluconeogenesis, glucagon.

INTRODUCTION

The adjuvant-induced arthritis is an experimental immunopathology in rats which shares many features of human rheumatoid arthritis (Rosenthal and Capetola, 1982). For this reason it is one of the most widely used models for evaluation of anti-inflammatory and antirheumatic drugs (Rainsford, 1982; Billingham, 1983). Furthermore, this model has also been used for studying metabolic alterations induced by rheumatoid arthritis. Caparroz-Assef et al., 1998 and Fedatto Jr. et al., 1999, for example, have recently shown that livers from adjuvant-induced arthritic rats present lower rates of gluco-neogenesis from a variety of substrates, including lactate plus pyruvate. It was also shown that glucose uptake is increased in livers from arthritic rats due to higher glucokinase activities (Fedatto Jr. et al., 2000). Inflammation also affects Ca\(^{2+}\) fluxes in hepatocytes. Barrit and Whitehouse (1977), for example, found that mitochondria isolated from inflamed rats exhibit a decreased ability to retain accumulated Ca\(^{2+}\) and a more rapid and greater degree of Ca\(^{2+}\) induced osmotic swelling. Moreover, Somasundaram and Sadique (1986) found that mitochondria from inflamed rats exhibit a reduction in Ca\(^{2+}\) uptake. Ca\(^{2+}\) movements and redistributions, on the other hand, are frequent and important events during hormone actions. Even glucagon, a hormone that acts predominantly via cyclic AMP, is also influenced by Ca\(^{2+}\). The latter seems to be especially involved in gluconeogenesis stimulation by glucagon, as shown by Silva et al., 1997. In the
perfused rat liver the Ca$^{2+}$ dependencies of the simultaneous gluconeogenesis and respiration stimulations by glucagon are strongly reduced at a lactate to pyruvate ratio of 100. At the more physiologic lactate to pyruvate ratio of 10, the Ca$^{2+}$ dependencies of gluconeogenesis and respiration stimulations are less pronounced, but still evident. It should be mentioned that glucagon increases the activity of several mitochondrial dehydrogenases and other enzymes, an action that is believed to be caused by Ca$^{2+}$ accumulation (McCormack, 1985; Quinlan and Halestrap, 1985; Walajtys-Rhode et al., 1992; Deaciuc et al., 1992).

Both phenomena, changes in Ca$^{2+}$ movements in the liver of arthritic rats and the Ca$^{2+}$ dependence of the glucagon action, immediately raise the question about the role of Ca$^{2+}$ in hepatic gluconeogenesis stimulation by glucagon under the former conditions. An answer to such a question can only be obtained by experimental means. For this reason, we have decided to measure gluconeogenesis from lactate plus pyruvate in livers from arthritic rats, in the presence and absence of Ca$^{2+}$. These experiments should bring an answer to the question whether Ca$^{2+}$ affects, or not, the action of glucagon on hepatic gluco-neogenesis in arthritic rats.

MATERIALS AND METHODS

Materials. The liver perfusion apparatus was built in the workshops of the University of Maringá. Crystalline glucagon was purchased from “Eli Lilly do Brasil”. All enzymes and coenzymes used in the enzymatic assays were purchased from “Sigma Chemical Co.” (St. Louis, USA). All other chemicals were from the best available grade (98-99.8% purity).

Animals. Male albino rats (Wistar), weighting 200-250 g, were fed ad libitum with a standard laboratory diet (Purina®, São Paulo, Brazil). For the induction of adjuvant arthritis, the animals were injected in the left hind paw with 100 µl of heat inactivated Mycobacterium tuberculosis suspended in mineral oil at a concentration of 0.5% (w/v). Two weeks after the induction of the disease, the animals showing characteristic arthritic lesions were selected for the experiments (Pearson, 1956). Rats with similar ages served as controls. All rats were starved for 24 hours before the surgical removal of the liver.

Liver perfusion. For the surgical procedure the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Hemoglobin-free, non-recirculating perfusion was done. The surgical technique was that one described by 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. 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.

Ca$^{2+}$-free perfusion. 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 0.2 mM ethylenediamine tetraacetate (EDTA). In order to ensure maximal depletion of the intracellular Ca$^{2+}$ pools, phenylephrine (2 µM) was infused repeatedly (3 times) during short periods of 2 minutes, with intervals of 5 minutes. According to Reinhart et al. (8), this procedure depletes the intracellular Ca$^{2+}$-pools which are normally mobilized when hormones are infused.

Analytical. Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their glucose contents by means of a standard enzymatic procedure (Bergmeyer and Bernt, 1974) The oxygen concentration in the outflowing perfusate was monitored continuously, employing a teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate (Kelmer-Bracht et al., 1984).

Calculations and treatment of data. The mean rates of glucose production ($\bar{G}_s$) during substrate infusion and before glucagon infusion were calculated according to the following equation:

$$\bar{G}_s = \frac{\int_{t_0}^{t_1} [G_s(t) - G_b] \, dt}{t_1 - t_0} \quad [1]$$
In equation [1] $t_0$ represents the time in which substrate infusion was started and $t_1$ the time at which glucagon infusion was initiated, $G_i(t)$ are the rates of glucose production and $G_b$ the small basal rate of glucose release (before substrate infusion). The mean rates of glucose release during glucagon infusion ($\overline{G_g}$) were calculated according to the relation:

$$\overline{G_g} = \frac{\int_{t_1}^{t_2} [G_i(t) - G_b] \, dt}{t_2 - t_1} - \overline{G_s}$$  \hspace{1cm} [2]

In equation [2] $G_i(t)$ are the experimental rates of glucose release during glucagon infusion, $t_1$ the time at which glucagon infusion was initiated and $t_2$ the time at which glucagon infusion was stopped.

The integrals in equations [1] and [2] were calculated analytically after fitting the experimental data to cubic spline functions. These calculations were done by means of the *scientist program* from MicroMath Scientific Software.

The statistical significance of the differences between parameters was evaluated by means of the Student-Newman-Keuls test after variance analysis; $p < 0.05$ was adopted as a criterion of significance.

**RESULTS**

Figures 1 and 2 illustrate the experimental protocol and the results obtained in the perfusion experiments. Figure 1 shows the glucose production and oxygen uptake measurements when the normal Krebs/Henseleit-bicarbonate buffer was employed; and Figure 2 illustrates the same measurements in the Ca$^{2+}$-free experiments. As revealed by the horizontal bars, the substrates (lactate plus pyruvate) were infused alone during 20 minutes.
After this time, glucagon was infused during 30 minutes in the presence of these substrates. A lactate to pyruvate ratio of 10 was chosen because this is a more physiologic condition.

Figure 1A allows to compare the responses of glucose production and oxygen uptake in the presence of Ca\(^{2+}\) of both livers from normal and livers from arthritic rats. All rats were fasted for a period of 24 hours before the perfusion experiments. Under these conditions the glycogen levels are low and the contribution of glyco-genolysis to glucose release is minimal (Scholz and Bücher, 1965). This is revealed by the very small rates of glucose release in the pre-perfusion period (0 to 8 minutes). Glucose production in the liver of normal rats raised more rapidly after initiation of substrate infusion than in livers from arthritic rats. At the end of 20 minutes hepatic gluconeogenesis in normal rats was clearly superior to that in arthritic rats. This observation confirms previous findings (Fedatto Jr. et al., 1999). The introduction of glucagon at 28 minutes produced clear increases in glucose production in the normal as well as in the arthritic condition. Oxygen uptake, as revealed by Figure 1B, was similar in livers from normal and arthritic rats, although the latter presented a small tendency toward higher values. Under both conditions the infusion of substrates and the subsequent infusion of glucagon produced increments in oxygen uptake. Especially in the presence of glucagon, there was a tendency toward higher values in livers from arthritic rats but, unlike to what occurred with gluconeogenesis, the sizes of the standard errors do not allow a clear definition of the differences.

Figure 2 shows the time-courses of glucose production and oxygen uptake in livers from normal and arthritic rats in the absence of Ca\(^{2+}\). Absence of Ca\(^{2+}\) means perfusion with no added Ca\(^{2+}\) after exhaustion of the intracellular pools, as described in Materials and Methods. Gluconeogenesis in the livers of normal and arthritic rats still responded to substrates and glucagon infusion.
The influence of Ca2+ on gluconeogenesis stimulation

Table 1 - Mean rates of glucose production in livers from normal and arthritic rats: the action of glucagon in the presence and absence of Ca2+. The mean rates were calculated as described in Materials and Methods. The experimental conditions were those described in the legends to Figures 1 and 2.

<table>
<thead>
<tr>
<th>Animal condition</th>
<th>Ca2+ in the perfusate</th>
<th>Gluconeogenesis (µmol min⁻¹ g⁻¹)</th>
<th>Percent stimulation caused by glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before glucagon infusion (G)</td>
<td>Increment due to glucagon infusion (G)</td>
<td></td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td>2.5 mM</td>
<td>0.700±0.093*ª§</td>
<td>0.317±0.025*ª 45.2</td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td>Ca2+-free</td>
<td>0.510±0.057ª‡</td>
<td>0.214±0.007ª§ 41.9</td>
</tr>
<tr>
<td>Arthritic (n=5)</td>
<td>2.5 mM</td>
<td>0.330±0.044*</td>
<td>0.345±0.024ª 104.5</td>
</tr>
<tr>
<td>Arthritic (n=5)</td>
<td>Ca2+-free</td>
<td>0.286±0.028ª§</td>
<td>0.053±0.012ª‡ 18.5</td>
</tr>
</tbody>
</table>

In each column the symbols *, †, ‡, ¶ and § represent pairs of values differing statistically from each other according to the Student-Newman-Keuls test (p < 0.05).

The response of the liver of arthritic rats, however, was again lower, especially to glucagon, where it was clearly a transient one. Oxygen uptake of livers from arthritic rats also presented a transient response to glucagon, as revealed by Figure 2B. Figures 1 and 2 allow to appreciate the time courses of the changes produced by substrates and glucagon on gluconeogenesis in a qualitative way. A more rigorous quantitative analysis, however, can be done by means of the data in Table 1. In this table the mean rates of glucose production, calculated as described in Materials and Methods, are listed in addition to the percent stimulation caused by glucagon. In normal rats, hepatic gluconeogenesis was smaller in the absence of Ca2+, the same occurring with the increment due to glucagon, an observation which confirms previous results of our laboratory (Silva et al., 1997). In proportional terms, however, the increment caused by glucagon was similar. In arthritic rats, besides confirming the smaller rates of gluconeogenesis when compared to the normal state, a phenomenon that was already evident in Figures 1 and 2, Table 1 also shows that the action of glucagon was different in the presence and absence of Ca2+. In the presence of Ca2+, the greatest mean increment was found in comparison with all other conditions. In the absence of Ca2+, on the other hand, livers from arthritic rats produced the smallest mean increment caused by glucagon. The latter is valid in absolute as well as in relative terms.

DISCUSSION

The main conclusion allowed by the observations of the present work is that stimulation of hepatic gluconeogenesis by glucagon is rendered more dependent on Ca2+ in arthritic than in normal rats. The following combination of observations leads to this conclusion: a) hepatic gluconeogenesis stimulation by glucagon in the arthritic condition was superior in relative terms to the normal condition in the presence of Ca2+; b) in the absence of Ca2+, however, stimulation of gluconeogenesis by glucagon in the arthritic condition was the smallest one, in relative as well as in absolute terms. It should be added that the kinetics of gluconeogenesis changes was accompanied by a similar kinetics in the oxygen uptake changes. When the gluconeogenesis changes were only transient, as it happened in the arthritic condition and in the absence of Ca2+, the oxygen uptake changes followed a similar pattern. It is usually accepted that the increase in oxygen uptake due to glucagon infusion under conditions of gluconeogenesis is a consequence of the increased energy demands (Zwiebel and Scholz, 1986). Nonwithstanding, dissociations between both variables have been reported under some special conditions, as for example, in the presence of diltiazem (Bracht et al., 1999). The question that arises is whether the general conclusion stated above can be reconciled with previous data in the specialized literature. It is
believed that gluconeogenesis stimulation by glucagon depends largely on oxygen uptake stimulation via a Ca\textsuperscript{2+}-dependent stimulation of mitochondrial dehydrogenases (McCormack, 1985; Quinlan and Halestrap, 1985; Walajtys-Rhode et al., 1992; Deaciuc et al., 1992). In the arthritic condition, as already mentioned in the Introduction, the capacity of accumulating Ca\textsuperscript{2+} intramitochondrially is reduced (Barrit and Whitehouse, 1977; Somasundaram and Sadique, 1986). The apparent contradiction that hepatic gluconeogenesis stimulation by glucagon in arthritic rats is even more dependent on Ca\textsuperscript{2+} may be related to the redox potential of the NAD\textsuperscript{+}/NADH couple in these animals. Derbocio (1999) has shown that the mitochondrial NADH/NAD\textsuperscript{+} ratio in the liver of arthritic rats is approximately half that of normal rats, meaning thus a much more oxidized state. This could be the result of a diminished substrate supply or an enhanced activity of the respiratory chain. The latter is improbable, because respiration in the arthritic condition is not substantially different from that of the normal condition. Consequently, the reduced substrate supply is more probable. This means also lower substrate concentrations for the various dehydrogenases that, in turn, require a more pronounced stimulation when glucagon is introduced in order to cope with the necessities of gluconeogenesis. This is a situation which could explain the more pronounced dependence on Ca\textsuperscript{2+} in livers from arthritic rats, in spite of the fact that the capacity of accumulating this cation is reduced, although not eliminated (Barrit and Whitehouse, 1977; Somasundaram and Sadique, 1986). In the absence of Ca\textsuperscript{2+}, glucagon would be unable to induce the generation of sufficient extra amounts of reducing equivalents in order to enhance respiration and gluconeogenesis.

The latter seems to be a plausible explanation, but one should not exclude the possibility that the phenomenon detected in this work depends also on cytosolic or intracellular membrane-bound factors. There are several circulating and cellular factors that are changed in the arthritic condition as, for example, tumour-necrosis factor alfa (TNF\textsubscript{a}), interleukin and stress hormones (Lee et al., 1987; Hellerstein et al., 1989; Roubenoff et al., 1994; Roubenoff et al., 1997). These and other factors could be modifying enzyme activities. For example, an increased activity of both phosphofructokinase and fructose bisphosphate phosphatase has been demonstrated in cultured myocytes treated with the cytokine cachetin/TNF\textsubscript{a} (Zentella et al., 1993). These enzymes are among those that are controlled by glucagon. Consequently, the possibility of a differential action of glucagon on enzymatic activities changed during the arthritic condition should not be ruled out and deserves further investigations.

**RESUMO**

O Ca\textsuperscript{2+} participa do estímulo da neoglicogênese hepática pelo glucagon e há também indicações de que fluxos de Ca\textsuperscript{2+} são modificados em ratos artríticos. Estes dados permitem levantar a questão de se a neoglicogênese hepática em ratos artríticos responde diferentemente ao glucagon e ao Ca\textsuperscript{2+}. O sistema experimental foi o fígado em perfusão isolada. Na presença de Ca\textsuperscript{2+}, o estímulo da neoglicogênese hepática pelo glucagon em ratos artríticos foi quase igual a aquele encontrado em ratos normais em termos absolutos, mas maior em termos relativos (104,5 e 45,2%, respectivamente). Na ausência de Ca\textsuperscript{2+}, no entanto, o estímulo da neoglicogênese hepática pelo glucagon em ratos artríticos foi menor em termos absolutos e relativos (18,5 e 41,9%, respectivamente). Pode-se concluir que o componente Ca\textsuperscript{2+}-dependente do estímulo da neoglicogênese pelo glucagon é mais importante em ratos artríticos do que em ratos normais.

**REFERENCES**


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