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Purinergic Effects of a Hydroalcoholic *Agaricus brasiliensis* (*A. blazei*) Extract on Liver Functions

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The effects of a hydroalcoholic extract of *Agaricus brasiliensis* (*A. blazei*) on functional parameters in the perfused rat liver were examined with emphasis on its content of nucleotides and nucleosides. Several nucleosides and nucleotides were identified in the *A. brasiliensis* extract, which was active on several liver functions. A significant part of the effects is the result of the purinergic action of nucleosides and nucleotides: pressure increment, glycogenolysis stimulation, transient inhibition of oxygen consumption, and redox state changes. Other phenomena such as the stimulation of gluconeogenesis, ureogenesis, and oxygen consumption are more likely consequences of the metabolic transformation of substrates contained within the extract, especially amino acids. It seems apparent that consumption of *A. brasiliensis* represents not only the ingestion of metabolic precursors but also the ingestion of substances that, even at low concentrations, can exert important signaling functions in the liver as well as in the organism as a whole.

KEYWORDS: ***Agaricus brasiliensis*; extracts; nucleosides; nucleotides; purinergic action**

INTRODUCTION

Edible mushrooms have long been appreciated for their flavor and texture and are recognized as an important source of bioactive compounds of medicinal value (*1*, *2*). Mushrooms are a valuable food, low in calories and high in minerals, essential amino acids, vitamins, and fibers. Studies have demonstrated that the regular consumption of mushrooms is beneficial to health, and they can be considered as functional foods or nutraceutical products (*3*).

Agaricus brasiliensis Wasser & Didukh, formerly known as *Agaricus blazei* Murril ss. Heinemann, is a basidiomycete popularly known in Brazil as Cogumelo do Sol and Cogumelo Piedade. It is widely used today as an edible mushroom and as a natural therapy in the form of a medicinal extract mostly for the prevention and treatment of a series of diseases. In accordance with Brazilian tradition, it would be useful against a variety of diseases such as diabetes, atherosclerosis, hepatitis, hypercholesterolemia, and heart disease (*4*). A β-glucan isolated from *A. brasiliensis* has been described to stimulate proliferation of lymphocyte T-cells in mice (*5*), to exhibit antitumor and antimicrobial activities in animals (*6*), and to protect against chemically induced DNA damage in human lymphocytes (*7*). In addition to β-glucan, edible mushrooms contain several supposedly bioactive compounds such as terpenoids, steroids, phenols, polyketides, quinazoline, polysaccharides, chitin, lectin, and proteins (*7*, *8*). Furthermore, nucleotides, nucleosides, and nucleobases have been isolated from several mushrooms, for example, *Ganoderma lucidum* (*10*), *Lentinus edodes* (*11*), *Cordyceps sinensis*, and *Cordyceps militaris* (*12*). Several of them are present at relatively high concentrations, raising the supposition that their functions go beyond the simple role of metabolic intermediates. In mammals this group of compounds is involved in the regulation and modulation of various physiological processes (*13*−*16*), so that they could be responsible for some of the physiologic effects normally attributed to edible mushrooms.

The liver is the metabolic organ *par excéllence*, and it is the first to receive most of the nutrients and substances absorbed in the intestine (*17*). In addition to its metabolic functions, exerted mainly by the hepatocytes, which in the liver transform many of the absorbed substances, the organ also responds in many ways to biological effectors (hormones or substances with hormone-like effects) by producing secondary effectors that can influence the physiology of the whole organism (*13*, *16*). It is thus of great interest to know what influence the substances contained within edible mushrooms can exert on the various liver functions. This interest concerns not only the normal physiology of the mushroom consumer but also the physiology of experimental animals subjected to experiments in which total or partial extracts of mushrooms are given orally to induce specific pharmacologic and physiologic responses. Despite this importance, studies in this field have rarely been done. Filling this gap is precisely the purpose of the present study in which the actions of a hydroalcoholic extract of *A. brasiliensis* were investigated on several parameters in the isolated perfused rat liver. The latter system allows one to measure several metabolic and functional parameters under fairly controlled conditions and has the great advantage that both microcirculation and cell-to-cell relationships are preserved (*16*, *17*). The perfusion experiments were preceded by the identification and quantification of the

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substances that might be responsible for the most important effects.

MATERIALS AND METHODS

Materials and Extract Preparation. The liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the enzymatic assays were purchsed from Sigma Chemical Co. (St. Louis, MO). All standard chemicals were of the best available grade.

Fruiting bodies (basidiocarps) of *A. brasiliensis* were obtained from a local producer in Maringá, PR, Brazil, in the spring of 2008. The young basidiocarps (cap closed) were harvested and dried. The basidiocarps were milled until a fine powder was obtained. The samples (50 g) were extracted by stirring with 1000 mL of ethanol (v/v) 70% at room temperature and at 130 rpm for 3 h and filtered through Whatman no. 1 paper. The extraction was repeated two times. No increase in yield was achieved by further extractions. The combined filtrates were concentrated with a rotary vacuum evaporator at 40 °C to eliminate ethanol and finally freeze-dried. The freeze-dried powder (yield = 50%) was stored in freezer until use.

Liver Perfusion and Analytics. Male albino rats (Wistar), weighing 180−220 g, were fed ad libitum with a standard laboratory diet (Purina). For the surgical procedure of liver isolation, the rats were anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg). The criterion of anesthesia was lack of body or limb movement in response to a standardized tailclamping stimulus. Animal handling and experiments were done in accordance with worldwide accepted ethical guidelines for animal experimentation. Hemoglobin free, nonrecirculating perfusion was performed (*17*). After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. 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 flow, provided by a peristaltic pump, was between 30 and 33 mL/min. When perfused with substrate-free perfusion medium, livers from 24 h fasted rats respire mainly at the expense of endogenous fatty acids (*17*). The freeze-dried extract was directly dissolved into the perfusion fluid at the desired concentration (up to 400 mg/L). Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite contents.

In the effluent perfusion fluid the following compounds were assayed by means of standard enzymatic procedures: glucose, lactate, pyruvate, urea, and ammonia (*18*). 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 (*17*). Metabolic rates were calculated from input−output differences and the total flow rates and were referred to the wet weight of the liver.

The portal perfusion pressure was monitored by means of a pressure transducer (Hugo Sachs Elektronic-Harvard Apparatus GmbH, March-Hugstetten, Germany). The sensor was positioned near the entry of the portal vein, and the transducer was connected to a recorder (*19*). The pressure changes were computed from the recorder tracings and expressed as millimeters of mercury (mmHg).

Chromatographic Procedures with the *A. brasiliensis* Extract. The hydroalcoholic extract of *A. brasiliensis* was fractionated by means of high-performance liquid chromatography (HPLC). The HPLC system (Shimadzu, Japan) consisted of a system controller SCL-10AVP, two pumps, model LC10ADVP, a column oven, model CTO-10AVP, and a UV−vis detector, model SPD-10AVP. A reversed-phase C18 HRC-ODS column (5 μm; 150 × 6 mm i.d.; Shimadzu) protected with a GHRC-ODS precolumn (5 μm; 10 × 4 mm i.d.; Shimadzu) was used with a gradient from reversed-phase 0.044 mol/L phosphate buffer solution, pH 6.0, to 0.044 mol/L phosphate buffer solution plus methanol (1.1), pH 7.0. The gradient was, in percent methanol, 0 min, 0%; 2.5 min, 0.25%; 5 min, 1.5%; 7 min, 2.5%; 8 min, 6%; 10 min, 7.5%; 12 min, 10%; 20 min, 15%; 28 min, 0%. The temperature was kept at 35 °C, and the injection volume was 20 μL, with a flow rate of 0.8 mL/min. The freeze-dried extract was dissolved in water. Identification of the peaks of the investigated compounds was carried out by comparison of their retention times with those obtained by injecting standards in the same conditions, as well as by spiking *A. brasiliensis* samples with stock standard solutions. The concentrations of the identified compounds in the extract samples were calculated by means of the regression parameters obtained from calibration curves. The calibration curves were constructed by separating chromatographically standard solutions of the compounds. Linear relationships were obtained between the concentrations and areas under the elution curves.

Other Analytical Procedures with the *A. brasiliensis* Extract. The total and reducing carbohydrates were determined by using the anthrone (*20*) and dinitrosalicylic methods (*21*), respectively, and expressed as glucose equivalents. Total phenolic compounds were quantified as described in ref *9* and expressed as gallic acid equivalents. The determination of flavonoids was done by means of a colorimetric assay (*22*) and expressed as catechin equivalents. Free amino compounds were measured using the ninhydrin method with L-alanine as standard (*23*). Alcohols, sorbitol, urea, and ammonia were quantified using standard enzymatic procedures (*18*).

RESULTS

Chemical Characterization of the *A. brasiliensis* Extract. Chemical, enzymatic, and chromatographic analyses were done to investigate the composition of the hydroalcoholic extract of *A. brasiliensis*. **Figure 1** shows a typical HPLC chromatogram of the hydroalcoholic extract with UV monitoring at 254 nm. There are numerous components absorbing at this wavelength. Several of them, however, could be identified by the usual techniques, which include comparison of retention times with those of standards and spiking of the *A. brasiliensis* samples with the same standards. It is clear that nucleotides, nucleosides, and nucleobases are important components of the *A. brasiliensis* extract. Quantification of the identified compounds is shown in **Table 1**. Adenosine was the most abundant followed by uridine.

Results of further analyses on the composition of the *A. brasiliensis* extract are also listed in **Table 1**. The content of total carbohydrates was relatively high, but that of reducing sugars very low (total/reducing = 1571). Polysacharides, including β-glucan, can be excluded because they are not extracted by solvents containing high proportions of ethanol. Among the nonreducing carbohydrates, sorbitol (which is readily metabolized by the liver) gives a very small contribution. Alcohols reactive to alcohol dehydrogenase, on the other hand, were detected at higher concentrations. This could include some residual ethanol from the extraction procedure. Much more significant is the concentration of ninhydrin-reactive amines. The detection of this class of substances corroborates previous observations about the presence of amino acids and other amines in several mushroom species including *A. brasiliensis* (*24*). In aqueous extracts of *A. brasiliensis*, urea is present at relatively high concentrations (*24*). In the hydroalcoholic extracts used in the present work, however, the urea concentration is much lower, well below the concentration of free ammonia, as revealed by **Table 1**.

Effects of the *A. brasiliensis* Extract on Hemodynamics and Oxygen Consumption. Several experiments were done to verify if the *A. brasiliensis* extract is active on the liver hemodynamics, because this kind of action is typical for nucleotides and nucleosides acting as purinergic agents (*13, 25*). In these experiments the extract was infused at a concentration of 400 mg/L during 20 min. With this extract concentration in the perfusion fluid, the total concentration of nucleotides plus nucleosides will reach at least 13.5 μM. This is a significant concentration in terms of purinergic activity (*13−16, 25−29*). **Figure 2A** shows that both perfusion pressure and oxygen consumption were stable before initiation of the extract infusion. An immediate response occurred, however, when the infusion was initiated. The perfusion pressure rapidly increased with a peak increment of 7.9 ± 1.1 mmHg at 2 min after infusion was begun. It declined progressively, however, and at the end of the infusion, it was only 1.4 ± 0.4 mmHg above the basal

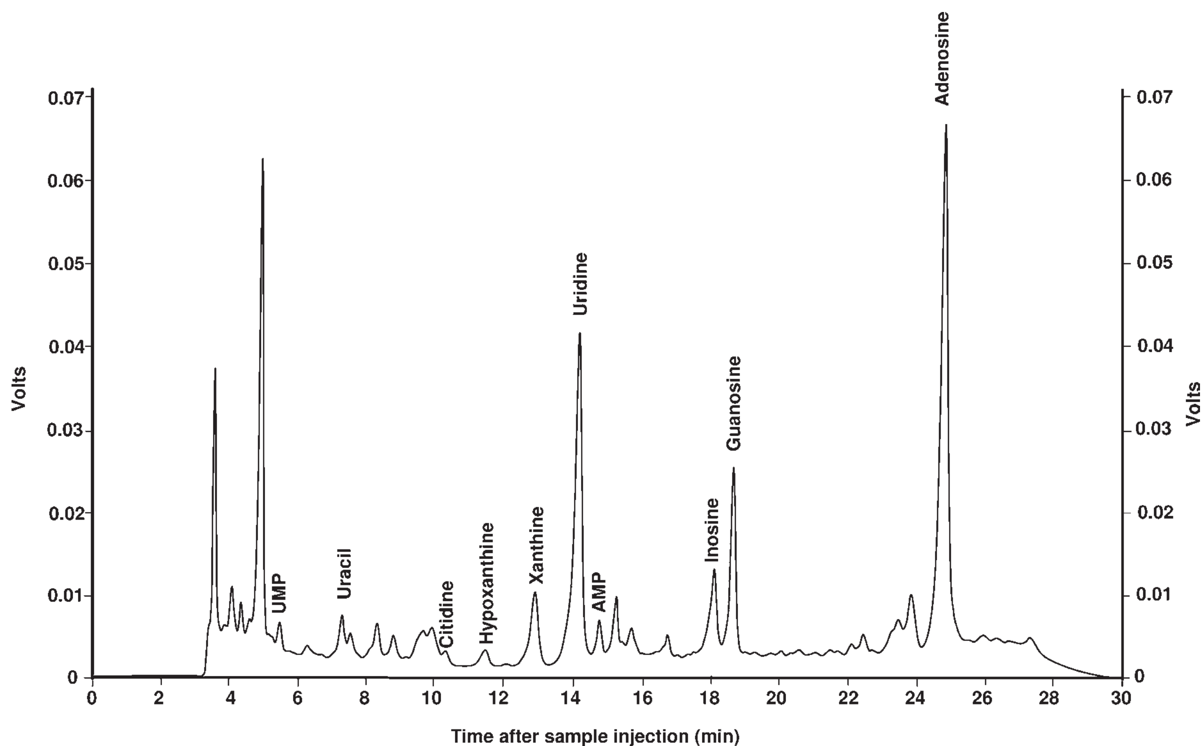

Figure 1. Typical chromatogram of an *Agaricus brasiliensis* extract. HPLC fractionation with spectrophotometric detection at 254 nm was performed as described under Materials and Methods. The concentration of the hydroalcoholic extract was 5 μg/mL, and the injection volume was 20 μL. The identified compounds are indicated.

Table 1. Chemical Composition of the Hydroalcoholic Extract of *A. brasiliensis*, as Determined by HPLC, Chemical, and Enzymatic Assays

kind of determination	compound	content (nmol/mg of extract)
HPLC	adenosine (*n* = 3)	14.40 ± 0.38
	cytidine (*n* = 3)	2.00 ± 0.13
	guanosine (*n* = 3)	2.96 ± 0.02
	uridine (*n* = 3)	10.82 ± 0.24
	inosine (*n* = 3)	1.96 ± 0.14
	adenosine 5′-monophosphate (AMP) (*n* = 3)	0.94 ± 0.24
	uridine 5′-monophosphate (UMP) (*n* = 3)	0.60 ± 0.01
	uracil (*n* = 3)	1.39 ± 0.02
	xanthine (*n* = 3)	2.36 ± 0.09
	hypoxanthine (*n* = 3)	0.72 ± 0.04
chemical assays	total carbohydrates (*n* = 5)	172.9 ± 7.1[a]
	reducing carbohydrates (*n* = 3)	0.11 ± 0.01[a]
	amino acids and other ninhydrin reactive amines (*n* = 3)	1810 ± 90[b]
	total phenolics (*n* = 3)	259.2 ± 7.7[c]
	flavonoids (*n* = 3)	23.6 ± 0.1[d]
enzymatic assays	ammonia (*n* = 3)	147.3 ± 17.0
	urea (*n* = 3)	47.0 ± 7.3
	ethanol and other alcohol dehydrogenase reactive compounds (*n* = 3)	157.9 ± 0.70
	sorbitol and other sorbitol dehydrogenase reactive compounds (*n* = 4)	13.3 ± 0.76

[a] Nanomoles of glucose equivalents. [b] Nanomoles of alanine equivalents. [c] Nanomoles of gallic acid equivalents. [d] Nanomoles of catechin equivalents.

levels. The response of oxygen consumption was relatively complex. There was an initial transient decline, which was followed by a gradual stimulation, which tended to a new steady state (0.55 ± 0.01 μmol min^{-1} g^{-1} above the basal rate) during the rest of the infusion time. Both effects were reversible; that is, cessation of the extract infusion resulted in termination of the effects.

Hemodynamic effects are basically a consequence of muscle contraction or distention, which are calcium-dependent phenomena. Another set of experiments was done to test a possible calcium dependence. In these experiments (**Figure 2B**) the livers were perfused initially with a calcium-free perfusion fluid. The extract infusion produced an immediate and stable increase in oxygen consumption without the oscillations seen during perfusion with a calcium-containing medium. The perfusion pressure was only minimally and transiently increased (1.43 ± 0.39 mmHg). The subsequent introduction of calcium did not produce significant alterations in the elevated oxygen consumption, but the perfusion pressure increased substantially with a peak value of 4.34 ± 1.65 mmHg and remained relatively elevated during the whole infusion time. Cessation of the extract infusion tended to restore the basal levels of both oxygen consumption and perfusion pressure.

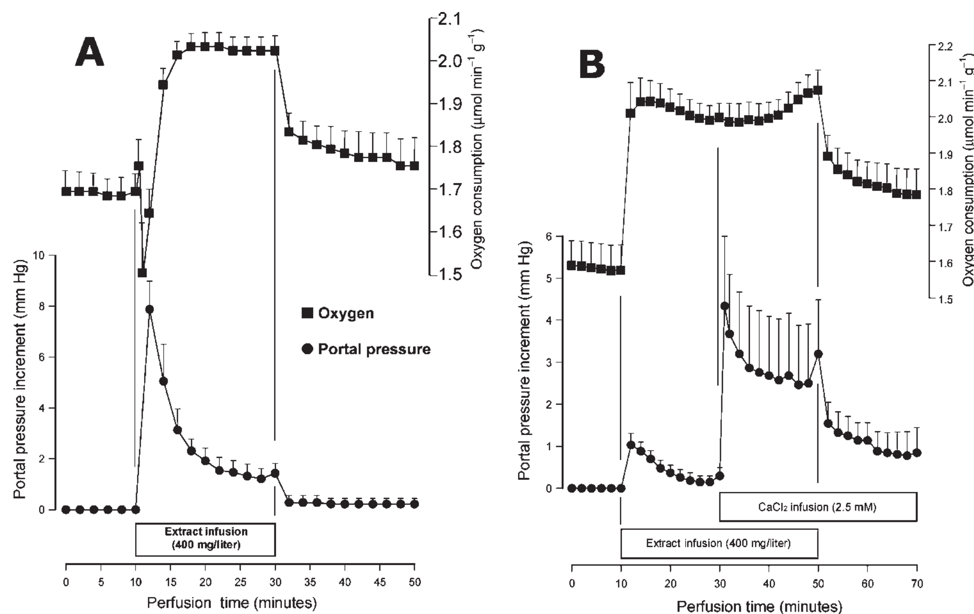

Figure 2. Actions of the hydroalcoholic *Agaricus brasiliensis* extract on oxygen consumption and portal perfusion pressure. Livers were perfused as described under Materials and Methods. In the experiments of panel **A** the normal Ca^{2+}-containing perfusion fluid was used. In the experiments of panel **B** the liver Ca^{2+} was absent initially, but it was introduced at 30 min of perfusion time. The extract was infused as indicated. The perfusion pressure was monitored by means of a pressure transducer. Values were subtracted from the basal perfusion pressure. Oxygen consumption was monitored polarographically. Data points ± mean standard errors are from four (**A**) or six (**B**) liver perfusion experiments.

Stimulation of oxygen uptake by the *A. brasiliensis* extract could be occurring in the mitochondria, in the microsomal electron transport chain, or in both. A simple way of testing these possibilities is to block the mitochondrial respiratory chain with cyanide (*17*). If stimulation persists, it will not be occurring in the mitochondria. To verify this, experiments were done in which 2 mM cyanide was infused, a concentration that completely blocks the mitochondrial respiratory chain (*17*). Under these conditions the respiratory rate of the liver was reduced to 0.79 ± 0.02 μmol min^{-1} g^{-1}, which corresponds to the activity of the microsomal electron transport chain plus the loss of oxygen by diffusion. Under these conditions the *A. brasiliensis* extract (400 mg/L) was practically inactive on oxygen consumption. The barely detectable increment was only 0.03 μmol min^{-1} g^{-1}, strongly indicating a mitochondrial origin of the extract effects on oxygen consumption.

Effects of the *A. brasiliensis* Extract under Glycogenolytic Conditions. Stimulation of oxygen uptake by the *A. brasiliensis* extract is a metabolic effect, and its presence raises the question about additional effects. Livers from fed rats when perfused with substrate-free medium survive at the expense of glycogen degradation via glycolysis and oxidation of endogenous fatty acids (*17*). In addition to oxygen consumption, **Figure 3** also shows the changes caused by the extract in several parameters related to glycogen catabolism: glucose release, lactate production, and pyruvate production. Glucose release was transiently increased with a peak value 150% above the basal values. Lactate production was also transiently increased with a peak value 75% above the basal rates. Pyruvate production, finally, was decreased by 74% and remained so during the whole infusion time. As a consequence of the increased lactate production and the decreased pyruvate production, the lactate to pyruvate ratio was considerably increased during the extract infusion. Due to the lactate dehydrogenase equilibrium in the liver cells, this parameter is an important indicator of the redox state of the cytosolic NAD^{+}− NADH couple (*17*), meaning, thus, that the extract increases the cytosolic NADH/NAD^{+} ratio.

The effects shown in **Figure 3** were further investigated for their concentration dependence. The mean results are shown in **Figure 4**. For glucose and lactate the peak increments were represented against the extract concentration. For pyruvate production and oxygen consumption, the steady-state decreases and stimulations, respectively, were represented. **Figure 4** shows almost linear concentration dependences in the range up to 400 mg/L for glucose, lactate, and pyruvate productions (panel **A**), with relatively small actions at the concentration of 100 mg/L. Oxygen consumption (panel **B**), however, was already elevated at the concentration of 100 mg/L. It increased somewhat at the concentration of 200 mg/L and declined again at the concentration of 400 mg/L.

Some of the metabolic effects described above can also be produced by ethanol and other alcohols, especially oxygen consumption stimulation and diminution of pyruvate release due to the establishment of reducing conditions (*30*). On the basis of the data in **Table 1** the alcohol concentration generated by 400 mg of extract/L will be around 60 μM. For this reason control experiments similar to those shown in **Figure 3** were done. In these experiments (not shown), 60 μM ethanol did not produce any changes in oxygen consumption, glucose release, and lactate production. A small decrease in pyruvate production, however, was found (12%).

Effects of the *A. brasiliensis* Extract under Gluconeogenic Conditions. The experiments shown in **Figure 5** were planned to investigate a possible action of the extract under gluconeogenic conditions. Livers from fasted rats were used. Their low glycogen content minimizes interference by glycogen catabolism. The small rates of glucose and urea release were mainly due to the transformation of endogenous amino acids (first 10 min in **Figure 5**). Oxygen uptake, however, was high due to endogenous fatty acid oxidation. The introduction of the extract at 10 min of perfusion time produced the usual increase in oxygen consumption and stable increases in the productions of urea and glucose (+0.30 and +0.12 μmol min^{-1} g^{-1}, respectively). The inflowing urea, due to its presence in the *A. brasiliensis* extract (**Table 1**), was equal to

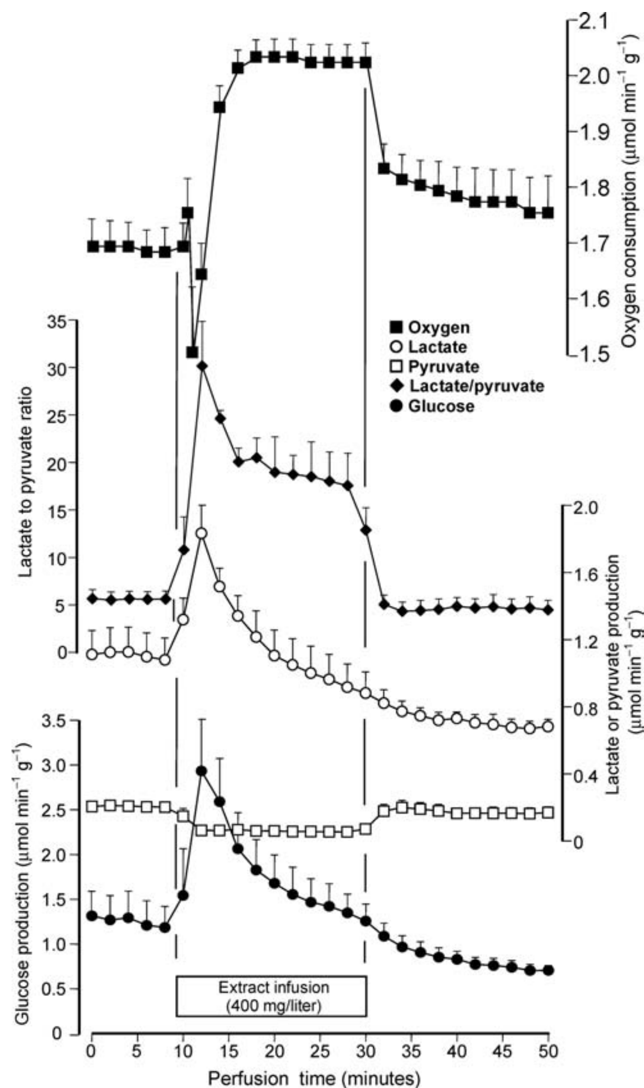

Figure 3. Changes in glycogen catabolism and oxygen uptake caused by the *Agaricus brasiliensis* extract. Livers of fed rats were perfused as described under Materials and Methods. Samples of the effluent perfusate were taken for measuring glucose, lactate, and pyruvate. Oxygen in the effluent perfusion fluid was monitored polarographically. Data are means ± mean standard errors of three liver perfusion experiments.

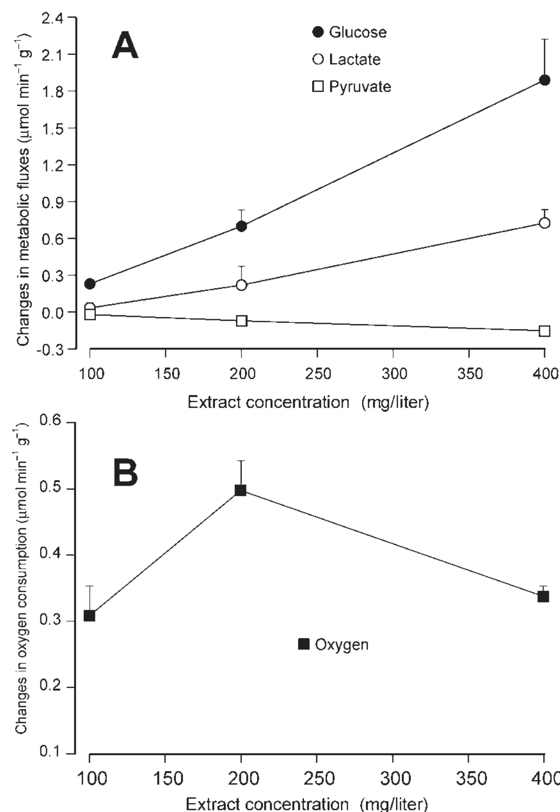

Figure 4. Concentration dependences of the changes in glycogen catabolism (**A**) and oxygen uptake (**B**) caused by the *Agaricus brasiliensis* extract. Data are from experiments of the kind illustrated by **Figure 3**. The changes in glucose and lactate production were calculated by subtracting the corresponding basal rates (before extract infusion) from the peak values just after initiation of the extract infusion. The changes in oxygen consumption and pyruvate production were calculated as the difference between the basal rates and the new steady-state rates during the extract infusion. Data are the means ± mean standard errors of three liver perfusion experiments for each extract concentration.

0.080 μmol min^{-1} g^{-1}. This value was subtracted from the output, so that urea production in **Figure 5** actually represents net production of this metabolite during the extract infusion. The rate of ammonia infusion due to its presence in the extract, on the other hand, was equal to 0.25 μmol min^{-1} g^{-1}. Output was lower, so that a substantial part of this ammonia was taken up with the kinetics shown in **Figure 5**.

In **Figure 6** the effect of the extract was investigated under conditions where gluconeogenesis was highly active due to the infusion of 2 mM lactate. The infusion of 2 mM lactate produced immediate increases in all parameters. At 30 min of perfusion time they had already stabilized at new steady states. The infusion of the extract (400 mg/L) produced several changes. Pyruvate production was strongly reduced (−83%) and remained so during the whole infusion time. Oxygen uptake experienced a clear and very pronounced initial decrease to values below the basal rate (−0.97 ± 0.07 μmol min^{-1} g^{-1}), which was followed by recovery and stimulation. Glucose production was transiently inhibited (38%), but recovered in parallel with the recovery in oxygen upake. The effects on pyruvate production and oxygen consumption were clearly reversible.

Effects of Inhibitors of Eicosanoid Production on the Actions of the Extract. Both the nature and kinetics of the effects of the *A. brasiliensis* extract suggest at least partly purinergic actions (*13*, *14*). This is especially true for the initial transient decreases in oxygen uptake and glycogenolysis stimulation (*13*, *25*, *27*). A characteristic of these effects is that they generally are at least partly mediated by eicosanoids, paracrine agents which in the liver are synthesized in Kupffer and endothelial cells and that exert their effects mainly in the neighboring hepatocytes (*13*, *16*, *25*). This possibility has been tested by measuring the action of two inhibitors of eicosanoid synthesis, namely, bromophenacyl bromide (*31*) and indomethacin (*32*). **Figure 7** shows the mean results that were obtained in the experiments in which livers from fed rats were utilized. Confirming previous observations, the compound did not affect in a significant way any of the analyzed parameters (*16*). When the extract was infused at a concentration of 400 mg/L, the responses of most variables were affected or modified. The scales used in **Figure 7** are the same as those in **Figure 3** to facilitate comparison. The initial and transient increases in glucose and lactate productions were almost abolished. The same occurred with the portal pressure increase, which was minimal in the presence of bromophenacyl bromide (0.86 ± 0.02 mmHg). The initial inhibition of oxygen uptake was absent, but the subsequent and stable increase was not modified. The decrease in pyruvate production was also present, although less pronounced (−50%). The lactate to pyruvate

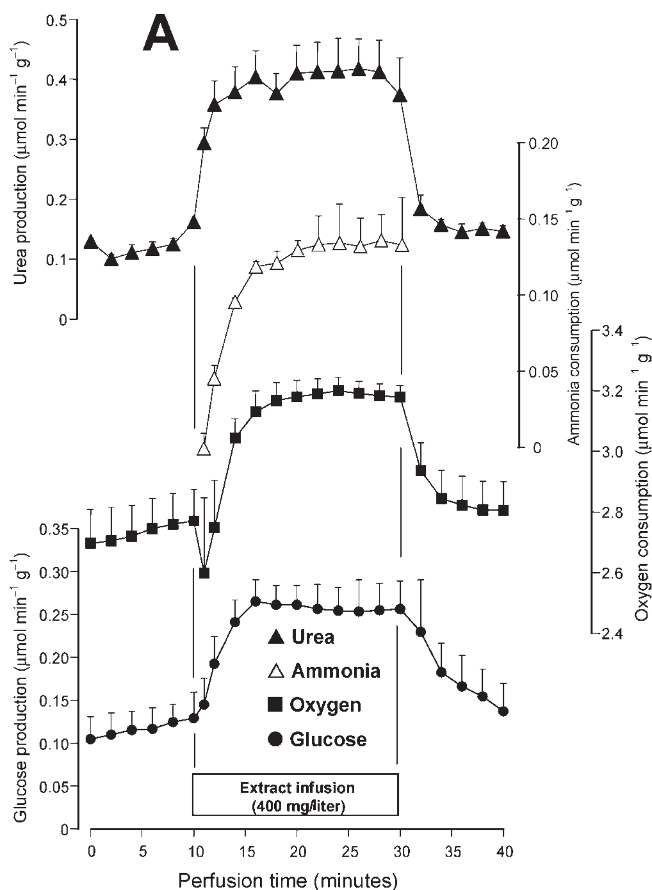

Figure 5. Changes in glucose production, urea production, ammonia production, and oxygen uptake caused by the *Agaricus brasiliensis* extract in livers from fasted rats. Livers from 24 h fasted rats were perfused as described under Materials and Methods. Substrate-free perfusion medium was used. The extract was infused at the indicated time at a concentration of 400 mg/L. Samples of the effluent perfusate were collected for metabolite assay. Oxygen in the outflowing perfusate was monitored polarographically. Data represent the means ± mean standard errors of three liver perfusion experiments.

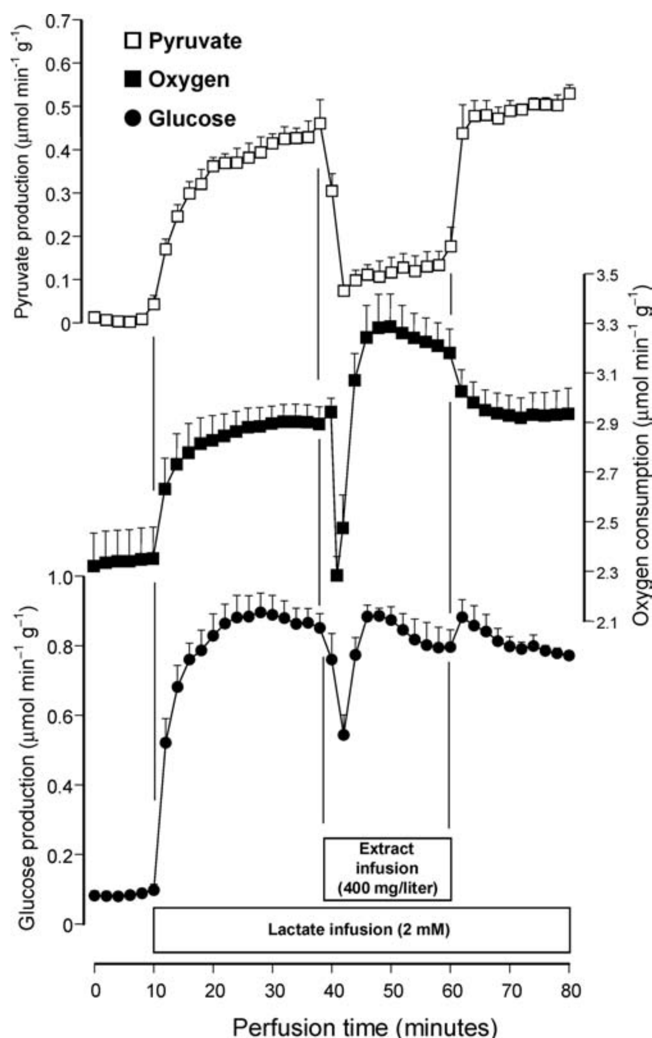

Figure 6. Changes in lactate gluconeogensis, pyruvate production, and oxygen uptake caused by the *Agaricus brasiliensis* extract in livers from fasted rats. Livers from 24 h fasted rats were perfused as described under Materials and Methods. Lactate (2 mM) was infused at the indicated time as a gluconeogenic substrate. The extract was infused at the indicated time at a concentration of 400 mg/L. Samples of the effluent perfusate were collected for metabolite assay. Oxygen in the outflowing perfusate was monitored polarographically. Data represent the means ± mean standard errors of three liver perfusion experiments.

ratio still suffered an increase, less pronounced, however, because no increase in lactate production was found. Additional experiments with livers from fasted rats (not shown) also revealed that bromophenacyl bromide abolished the transient inhibitions of oxygen uptake and gluconeogenesis caused by the extract. Oxygen uptake stimulation, however, was not affected. The actions of indomethacin (not shown), the other inhibitor of eicosanoid synthesis (*32*), were essentially the same as those of bromophenacyl bromide: abolition or diminution of all effects of the extract in livers from both fed and fasted rats with the exception of oxygen uptake stimulation.

Effects of Suramin on the Actions of the Extract. Experiments were also done with suramin, a purinergic receptor antagonist (*33*). The results of experiments with livers from fed rats are shown in **Figure 8**. At the concentration of 200 μM, suramin practically abolished the effects of the *A. brasiliensis* extract on glucose, lactate, and pyruvate productions. As a consequence, the increase in the lactate to pyruvate ratio was also absent. Furthermore, suramin reduced the peak increase in the perfusion pressure to 32% of the increment in the absence of the compound. The initial inhibition in oxygen consumption was also absent, but the stable increment was not affected.

DISCUSSION

The results of this work present evidence that the hydroalcoholic extract of *A. brasiliensis* is active on several liver functions in a relatively complex way. In the range up to 400 mg/L it affects hemodynamics, glycogenolysis, glycolysis, gluconeogenesis, ureogenesis, and the redox state of the cytosolic NAD^+-NADH couple. A significant part of these effects seems to be the result of purinergic action of several constituents of the extract. This includes the hemodynamic effects, the stimulatory actions on glycogenolysis (glucose and lactate release), the transient inhibitions of oxygen uptake and lactate gluconeogenesis, and the diminution of pyruvate production (meaning increased cytosolic $NADH/NAD^+$ ratios). Another set of phenomena, namely, the increased gluconeogenesis and ureogenesis during substrate-free perfusion of livers from fasted rats and especially the ubiquitous stimulation of oxygen consumption, are more likely a consequence of the metabolic transformation of several substrates contained within the extract, especially amine compounds, probably amino acids.

The conclusion that the extract exerts a purinergic action is based on a set of observations. First, the extract really contains several nucleotides and nucleosides that are known to exert

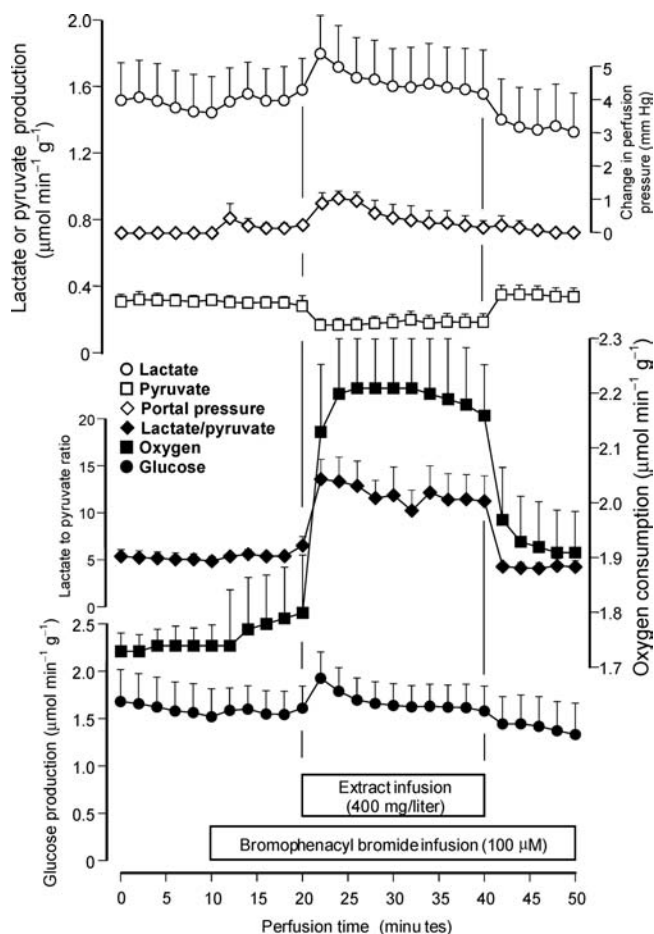

Figure 7. Influence of bromophenacyl bromide on the metabolic and hemodynamic effects of the *Agaricus brasiliensis* extract in livers from fed rats. Livers were perfused as described under Materials and Methods. The infusion of bromophenacyl bromide was started 10 min prior to the extract infusion and continued until the end of the experiment. The extract was infused from 20 to 40 min of perfusion time. Samples were taken for the measurement of glucose, lactate, and pyruvate. Oxygen was monitored polarographically. The portal perfusion pressure was monitored by means of a pressure transducer. Data represent the means ± mean standard errors of three liver perfusion experiments.

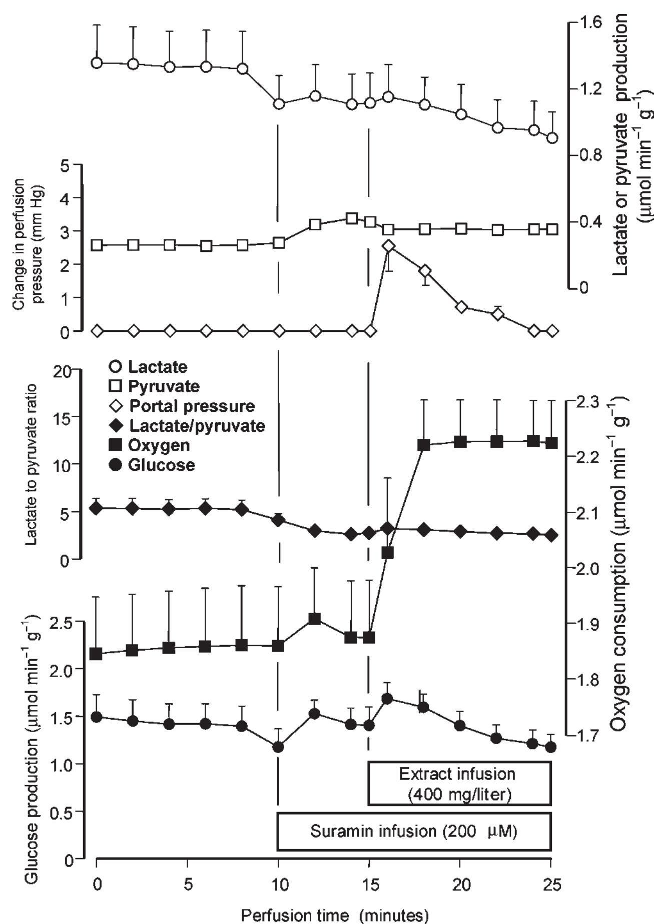

Figure 8. Influence of suramin on the metabolic and hemodynamic effects of the *Agaricus brasiliensis* extract in livers from fed rats. Livers were perfused as described under Materials and Methods. The infusion of suramin was started 5 min prior to the extract infusion and continued until the end of the experiment. The extract was infused from 15 to 25 min of perfusion time. Samples were taken for the measurement of glucose, lactate, and pyruvate. Oxygen was monitored polarographically. The portal perfusion pressure was monitored by means of a pressure transducer. Data represent the means ± mean standard errors of four liver perfusion experiments.

purinergic actions in the liver and other tissues (*13−16*). The concentration in the perfusion fluid was also compatible with the observed range in which these substances are usually active. Frequently their actions begin at 1 μM (*13*, *25−29*). Adenosine, for example, was found to stimulate glycogen phosphorylase at the concentration of 3.3 μM (*26*). In some cases, however, purinergic agents can act at much lower concentrations. For example, inosine, also present in the *A. brasiliensis* extract, has been reported to stimulate glycogenolysis and other parameters in the nanomolar range (*29*). The nature and kinetics of the effects of the purinergic agents in the liver are also fully compatible with the effects that were found in the present work. In this respect, the actions of a relatively great number of purinergic agents in the liver have already been extensively investigated, and their effects are quite similar (*13*, *14*, *16*). Adenosine, which is the most abundant nucleoside in the hydroalcoholic extract of *A. brasiliensis*, is also one of the most intensely investigated purinergic agents. In the perfused rat liver, it produces, at fairly low concentrations, practically the same transient inhibition of oxygen consumption and the same transtient increases of glycogenolysis and perfusion pressure as observed in the present work (*13*, *25*, *27*). Another argument in favor of a purinergic action of the *A. brasiliensis* extract is the sensibility to inhibitors of eicosanoid synthesis (*16*, *25*, *27*), a phenomenon that was also observed in the present work with two different inhibitors, namely, bromphenacyl bromide and indomethacin. The sensitivity of the purinergic action to inhibitors of eicosanoid synthesis is due to the fact that a substantial part of it is mediated by eicosanoids. In the liver these eicosanoids are produced in Kupffer and endothelial cells, but they exert their action mainly in hepatocytes. Adenosine, for example, has been found to stimulate the release of prostaglandin D_2 and thromboxane B_2 (*25*). It is likely, however, that several types of eicosanoids are released, so that the overall action is the result of the combined effects of several mediators. It should be stressed that infusion of eicosanoids into the liver in fact reproduces many of the effects elicited by purinergic agents (*34*). Finally, several of the extract effects found in this work were sensitive to suramin, a known antagonist of purinergic action, especially when P_2 purinergic receptors are involved (*33*). It is remarkable that suramin abolished even the action of the extract on pyruvate production, an effect that was only partly diminished by the inhibitors of eicosanoid synthesis. It is important to stress

that practically all nucleosides and nucleotides possess some purinergic activity in the liver so that the actions observed in the present work represent the combined effects of several compounds and not only the action of adenosine.

As already mentioned above, a considerable part of the actions of the *A. brasiliensis* hydroalcoholic extract cannot be attributed to purinergic agents. The most prominent action of this kind is oxygen consumption stimulation, which superimposes on the initial inhibition, which is probably of purinergic origin. Although nucleotides and nucleosides, as purinergic agents, can also stimulate oxygen consumption in the perfused rat liver, this usually occurs at concentrations that are higher than those occurring in the present study (*14*, *16*). With the *A. brasiliensis* hydroalcoholic extract, oxygen consumption stimulation was always present, irrespective of the metabolic conditions of the animal (fed or fasted), the presence of inhibitors, or the absence of calcium. Because oxygen consumption stimulation is of mitochondrial origin, as indicated by the experiments in which the mitochondrial respiratory chain was blocked with cyanide, it possibly represents the transformation/oxidation of substrates present in the *A. brasiliensis* extract. These substrates are not sugars, as free monosaccharides are virtually absent. Sorbitol, the only sugar–alcohol metabolized by the liver at high rates, can also be excluded due to its extremely low concentration in the extract (*35*). Amino acids, however, are a likely possibility. Amino acids increase oxygen consumption because they are oxidized via tricarboxylic acid cycle and respiratory chain and also because of an increased demand of ATP due to urea and glucose synthesis. In terms of alanine equivalents the content of ninhydrin reactive amines in the extract (**Table 1**) would correspond to a concentration near 720 μM in the perfusion experiments with 400 mg/L. Such a concentration of amino acids in the portal vein, although relatively low, already produces detectable changes in metabolic fluxes such as oxygen consumption, glucose synthesis, and ureogenesis, provided that appropriate conditions are used (*36*). These conditions are the virtual absence of other substrates, which can be achieved by substrate-free perfusion, and also the virtual absence of cellular glycogen, a condition that minimizes glucose release from endogenous sources. Under such conditions it was, in fact, possible to observe that, besides the usual increment in oxygen consumption, the extract produced a significant and stable increase in glucose production, which was accompanied by a substantial increase in urea production. Ammonia, also present in the extract, must also have contributed to urea synthesis. Its contribution, however, can be easily calculated on the basis of the rates of ammonia consumption. The steady-state rate of ammonia consumption attained at the end of the extract infusion was around 0.135 μmol min^{-1} g^{-1}, which accounts for only 23.5% of the total increment in urea production. Most urea must, thus, come from the transformation of amino acids contained within the extract. The increment in glucose production is expected by virtue of the presence of glycogenic amino acids in the extract (*24*). The fact that no increment in glucose production was found in the presence of lactate is understandable due to the fact that in the presence of this substrate the gluconeogenic pathway was already operating at its maximal or nearly maximal capacity. In this respect it should be mentioned that the increment in oxygen uptake caused by the extract in the presence of lactate was also considerably smaller than that found during substrate-free perfusion, probably because lactate itself had already produced a substantial increment.

The extract also contains a reasonable quantity of phenolics and a much smaller amount of flavonoids. From **Table 1** one can deduce that the infusion of 400 mg of extract/L resulted in a total phenolic concentration of approximately 100 μM (in gallic acid equivalents) and a total flavonoid concentration of approximately 9.5 μM (in catechin equivalents). Although it is clear that these classes of compounds have antioxidant activity, the latter usually has no short-term influence on the parameters that were measured in the present work (*37*). Gallic acid, as shown recently, exerts a small inhibitory effect on gluconeogenesis at the concentration of 100 μM (*37*). On the basis of literature data, however, its maximal concentration in the perfusion fluid would be equal to 40 nM (*38*), which is extremely low. Flavonoids are also known to be active on metabolism. Quercetin, for example, was shown to affect several metabolic pathways in the liver. However, it exerts these actions at concentrations well above 10 μM (*39*). All of these data strongly suggest that the influence of the flavonoids and phenolics present in the *A. brasiliensis* extract on the parameters measured in the present work was of minor importance. This conclusion, obviously, does not exclude the possibility that these compounds could be influencing other variables in the liver.

The results of the present work have implications for both the consumer of *A. brasiliensis* as a functional and nutraceutic food (*3*) and for the experimenter interested in the pharmacologic effects of the mushroom (*4*). It seems evident that consumption of *A. brasiliensis* represents not only the ingestion of metabolic precursors but also the ingestion of substances that, even at low concentrations, can exert important signaling functions not only in the liver but in the organism as a whole (*15*). Current evidence indicates that active nucleotides and nucleosides play a fundamental role in the rapid, precise, and highly specific control of cellular functions in practically all mammalian tissues, from the brain to the gastrointestinal tract. It is thus highly probable that many of the actions that have been attributed to mushrooms in general are at least partly mediated by purinergic agents. In this respect an interesting aspect of the ingestion of mushrooms is the presence of several different types of purinergic agents. Different but similar agents can act on different subtypes of receptors, a situation that increases substantially the possibility of cross-talking and synergisms.

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