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Influence of Subcutaneous Indole-3-Acetic Acid Administration in Metabolism and Function of the Rat Leukocyte

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Abstract: Indole-3-Acetic Acid (IAA) is a naturally occurring auxin, well known for its regulatory function in plant growth and its association with fruit ripening and senescence. This study aimed to investigate the effect of IAA administration on leukocytes metabolism and function. This growth plant hormone promoted a decrease in glucose oxidation (20%) by neutrophils and an increase in glutamine consumption (31%) by these cells, but no alteration was shown on consumption of the glucose and glutamine by lymphocytes. IAA did not cause a marked effect on the key enzyme activities of glucose and glutamine metabolism in neutrophils or lymphocytes. The IAA treatment did not show alteration of the phagocytic parameters of *Staphyloccocus aureus* engulment by neutrophils, with bacteria killed by myeloperoxidase activity in these cells, compared with the control animals. The observations presented led us to conclude that administration of subcutaneous IAA promotes an alteration in the rat's neutrophil metabolism, deduced by a decrease in glucose consumption and an increase in glutamine consumption in neutrophils; suggesting that the alteration of glucose metabolism could be compensated for by glutamine utilization in these cells. The metabolism alteration in rat neutrophils does not reduces the phagocytic capacity or myeloperoxidase activity of this cell. The effect of IAA administration, similar to *in vitro* studies, may reflect the reaction between IAA and myeloperoxidase.

Key words: IAA, plant growth regulation, ripening, neutrophil, lymphocyte, enzyme activity

INTRODUCTION

Indole-3-acetic acid (IAA) is the most abundant naturally occurring auxin, well known for its regulatory function in plant growth (Rapparini *et al*., 2002) and can be associated with fruit ripening and senescence (McDonald *et al*., 1997). Exogenous application of auxin has been proposed for plant growth regulation in agriculture to control pests and increase the production of a wide variety of crops (Mickel, 1978) and for a delay in fruit ripening (McDonald *et al*., 1997; Tingwa and Young, 1995; Vendrell, 1969; Frenkel and Dyck, 1973; Purgatto *et al*., 2001). Recent study showed a toxic effect of the IAA on animal's organisms and others have shown that exogenous IAA can have benefic effects such as an increase the engulfment of inert particles by neutrophils.

Animals can obtain IAA from intestinal absorption (Weissabach *et al*., 1959) or from synthesis from tryptophan in various tissues (Mills *et al*., 1991). Recent studies have showed that the plasma levels of IAA are elevated in human diseases such as phenylketonuria (Armstrong and Robinson, 1954), renal dysfunction (Bertuzzi *et al*., 1997) and insulin dependent diabetes mellitus (Rogerson *et al*., 1991).

One intravenous dose of IAA (higher than 50 mg of IAA per kg of body mass) is sufficient to promoting myotonia in mice, induces a delay in muscle relaxation in cats (Fuller *et al*., 1971) and to cause hypothermia and hypoglycemia (Mirsky and Diengott, 1957). Orally IAA administration (3 mg per each rat daily for 21 days) showed a toxic effect in rats, deduced by increased lipid peroxidation level and inhibition of antioxidant enzyme activities in various tissues (Celik *et al*., 2006a, b; Celik and Tuluce, 2006). On the other hand, the oral or subcutaneous administration of IAA (lower than 40 mg of IAA per kg of body mass during 14 days) can promote a possible beneficial effect available increasing the phagocytic capacity of rat neutrophils (Lins *et al*., 2006; Pugine *et al*., 2006) and does not show a prooxidant effect in neutrophils and lymphocytes (Pugine *et al*., 2006) and liver (Olivera *et al*., 2005).

Leukocytes such as neutrophils and lymphocytes play an important role in immune and inflammatory responses. These cells are of fundamental importance not

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only in preventing or limiting infection, but also in the overall process of repair and recovery from injury. These cells utilize glutamine at high rates in addition to glucose (Pithon-Curi *et al*., 2003). The processes of endocytosis, secretion of active compounds and generation of reactive oxygen species have been assumed to be mostly dependent on glucose and glutamine metabolism in neutrophils (Pithon-Curi *et al*., 1998).

The toxic effect of indole acetic acid in cultured neutrophils is associated with cell peroxidase activity (Melo *et al*., 1998) and these processes have been implicated for the activation of glucose and glutamine metabolism (Melo *et al*., 2004a). Neutrophils present a higher peroxidase activity than inflammatory macrophages, whereas in lymphocytes this enzyme activity is not present (Melo *et al*., 2004b). Neutrophils have a high Myeloperoxidase (MPO) activity, an enzyme present in azurophil granules and responsible by hypoclorous acid generation during the phagocytic process. IAA leads to marked ultra structural changes and death of cultured neutrophils (Melo *et al*., 1998), whereas the toxic effects of IAA on lymphocytes are only observed when exogenous peroxidase is added to the culture medium or when the cells are co-cultivated with neutrophils (Melo *et al*., 2004b). The combination of IAA and horseradish peroxidase is cytotoxic towards mammalian cells (Folkes *et al*., 1999).

The information above led us to study the effect of IAA on leukocytes metabolism. This study was undertaken to investigate the possible utilization of indole-3-acetic acid for animals. For this purpose, the effect of subcutaneous administration of IAA in rats was evaluated using the following parameters in neutrophils and lymphocytes: Glucose metabolism available by glucose consumption, lactate production and by enzymes activities such as hexokinase, citrate syntase, lactate dehydrogenase and glucose-6-phosphte dehydrogenase and glutamine metabolism available by glutamine consumption, glutamate production and by the phosphate-dependent glutaminase. The possible effect of IAA administration on the phagocytic capacity was evaluated on neutrophils exposed to *Staphylococcus aureus* and by determination of myeloperoxidase activity in neutrophils.

MATERIALS AND METHODS

All chemicals and enzymes were of analytical grade. IAA was obtained from Sigma Chemical Company (St. Louis, MO, USA). A stock solution of IAA was prepared in PBS and pH adjusted to 7.2 by using NaOH aqueous solution.

IAA preparation: A stock solution of IAA (10 mg mL^{-1}) was prepared in Phosphate-Buffered Saline (PBS) and NaOH (5 M) was added for complete solubility of this acid and thereafter the pH value was adjusted to 7.4 by using HCl (25%, v v^{-1}).

Animals and treatment: Male Wistar rats about 2 months of age, weighing 110-125 g, were obtained from the Department of Basic Science, Faculty of Animal Science and Food Engineering, São Paulo University, São Paulo, Brazil. Five groups with 4 or 6 animals each was studied including a control group and one group by subcutaneous IAA administration. The animals were maintained in a room at 23°C, in a light/dark cycle of 12/12 h.

The animals were fed *ad libitum* during 15 days and received for 14 days subcutaneous administration of indole-3-acetic acid. The control group received only PBS and the other group received indole acetic acid at 1.0, 2.0, 18.0 and 40.0 mg of IAA per Kg of body mass per day. Recent work was showed that these doses of IAA administration were sufficiently for increase the phagocytic capacity by rat´s neutrophils (Lins *et al*., 2006; Pugine *et al*., 2006) but, no information was performed about metabolic pathway in these cells. The rats were handled every day and so they got used to the treatment. This procedure was carried in accordance of Institutional Animal Ethics Committee.

Neutrophils preparations: Neutrophils were obtained by intraperitoneal (i.p.) lavage with 40 ml sterile PBS, 4 h after the i.p. injection of 20 ml sterile Oyster glycogen solution (Sigma, Type II) at 1% in PBS (Melo *et al*., 1998). Similar procedure was used by others (Mulligan *et al*., 1998). The number of viable cells, > 95%, was always counted in a Neubauer chamber in an optical microscope (Nikon, Japan), using a Trypan Blue solution at 1%.

Lymphocytes preparations: Lymphocytes were obtained from mesenteric lymph nodes as previously described (Melo *et al*., 1998). The cell suspensions were centrifuged (850 g during 8 min) 3 times in PBS. The number of viable cells, > 95%, was always counted in a Neubauer chamber in an optical microscope (Nikon, Japan), using a Trypan Blue solution at 1%. Lymphocytes (absent of mieloperoxidase) were included in this study for comparison with neutrophils (high myeloperoxidase activity) (Melo *et al*., 1998). The toxic effect of IAA is correlated with high myeloperoxidase activity (Melo *et al*., 1998).

Cell incubation: Neutrophils or lymphocytes (1.0×10^{6} cells mL^{-1}) were incubated for 1 h at 37°C in PBS with

2% (w v^{-1}) defatted Bovine Serum Albumin (BSA) in the presence of glucose (5 mM) or glutamine (2 mM). After incubation, the cells were disrupted by addition 0.2 mL 25% (w v^{-1}) perchloric acid. Protein was removed by centrifugation and the supernatant fluid was neutralized with 40% KOH solution and a Tris(hydroxymethyl-aminomethane)/KOH (0.5/2.0 M) solution for the measurement of the metabolites.

Metabolite measurements: Samples of the incubation cells were used for measurements of glucose (Barham and Trinder, 1972), glutamine (Windmueller and Spaeth, 1974), lactate (Engel and Jones, 1978) and glutamate (Bernt and Bergmeyer, 1974). Production or consumption of NADH or NADPH was monitored at 340 nm.

Enzyme assays: The activities of hexokinase (EC 2.7.1.1), citrate synthase (EC 4.1.3.7), glucose-6-phosphate dehydrogenase (EC 1.1.1.4) and phosphate-dependent-glutaminase (EC 3.5.1.2) were determined as previously described (Pithon-Curi *et al*., 2003). The extraction medium for hexokinase contained 50 mM Tris.HCl, 1mM EDTA, 30 mM $MgCl_2$ and 20 mM β-mercaptoethanol at pH 7.4. The extraction medium for citrate synthase and glucose-6-phosphate dehydrogenase contained 50 mM Tris.HCl and 1 mM EDTA; the final pH values were 7.4 and 8.0, respectively. The extraction medium for phosphate-dependent-glutaminase consisted of 150 mM phosphate buffer, 1.0 mM EDTA and 50 mM Tris.HCl at pH 8.6. The extraction medium for lactate dehydrogenase and peroxidase consisted of 10 mM phosphate buffer at pH 7.4. For all enzyme assays, Triton X-100 was added to the assay system (0.05% - v v^{-1}) to complete the extraction of the enzymes. The extraction medium for measurement of Myeloperoxidase (MPO) consisted of 50 mM potassium phosphate buffer, pH 6.0.

The final volume of assay mixture always was 1.0 mL. Citrate synthase was assayed by following the rate of change at 412 nm. Hexokinase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase were assayed by following the changes at 340 nm. Phosphate-dependent glutaminase was assayed as described by Curthoys and Lowry (Curthoys and Lowry, 1973). In this assay, glutamine is converted into glutamate by glutaminase during 10 min. Glutamate is then determined by using a spectrophotometric assay at 340 nm. All spectrophotometric measurements were performed in a Bekman-DU800 spectrophotometer. Preliminary experiments established that extraction and assay procedures produced maximum enzyme activities as described by Crabtree *et al.* (1979). The MPO activity was determined by measuring the oxidation of *o*-dianisidine in the presence of hydrogen peroxide at 460 nm. One unit of MPO was defined as that degrading 1 μmol of H_2O_2 min^{-1} at 25°C (Hillegass *et al*., 1990).

Protein determination: Protein content of leukocytes was measured by the method of Bradford (Bradford, 1976), using Bovine Serum Albumin (BSA) as standard.

Expression of results and statistical analysis: Comparisons between groups were initially tested by Analysis of Variance (ANOVA). The alpha level (significance level related to the probability of rejecting a true hypothesis) was set at 0.05. Significant differences were then compared using Tukey's Honestly Significant Difference Test with a significance coefficient of 0.05.

RESULTS

The effect of IAA administration on glucose and glutamine metabolism was investigated in neutrophils and lymphocytes from control and treated animals (Table 1). The acid promoted a decrease in glucose oxidation (by 20%) by neutrophils and an increase in glutamine consumption (31%) by these cells, but no alteration was observed with respective lactate and glutamate production. Indole acetic acid did not show an alteration in the consumption of glucose and glutamine and lactate and glutamate production by lymphocytes.

Indole-3-acetic acid did not cause a marked effect on the key enzyme activities of glucose and the glutamine metabolism of neutrophils (Table 1). Similarly, no effect was observed on the enzymes activities of lymphocytes, evaluated from analyzing treated animals and controls, respectively (Table 1).

Phagoytosis of *Staphylococus aureus* by neutrophils is accompanied by engulfment and killing (Table 1). The IAA treatment did not show a significant alteration in these parameters compared with the control animals. Similarly, myeloperoxidase activity in neutrophils did not show any alteration following IAA administration (Table 1).

The others doses (1, 2 and 18 mg of IAA per Kg of body mass per day) do no affect the glucose or glutamine metabolism on neutrophils and lymphocytes. Similarly, IAA administration at 18 mg per Kg of body mass per day did not alter the phagocytosis of S. *aureus* by rat's neutrophil.

Table 1: Effect of IAA subcutaneous administration on glucose and glutamine consumption, lactate and glutamate production and maximal activities of Hexokinase (HK), Citrate Synthase (CS), Glucose-6-Phosphate Dehydrogenase (G6PDh), Lactate Dehydrogenase (LDH) and Glutaminase (GLUT) by neutrophils and lymphocytes. Myeloperoxidase activity (MPO) in neutrophils and microorganism phagocytosis available by *S. aureus* ingestion and *S. aureus* killed by neutrophils from controls and treated animals

	Neutrophil		Lymphocyte	
	Control	Treatment	Control	Treatment
Glucose consumption ($\mu mol.mL^{-1}.(1.0\times10^{7}$ cells$)^{-1}$	0.51±0.07	0.41±0.03*	0.24±0.03	0.17±0.03
Glutamine consumption ($\mu mol.mL^{-1}.(1.0\times10^{7}$ cells$)^{-1}$	0.29±0.02	0.38±0.02*	0.47±0.04	0.35±0.05
Lactate production ($\mu mol.mL^{-1}.(1.0\times10^{7}$ cells$)^{-1}$	0.23±0.02	0.27±0.03	0.52±0.03	0.48±0.05
Glutamate production ($\mu mol.mL^{-1}.(1.0\times10^{7}$ cells$)^{-1}$	0.22±0.02	0.24±0.02	0.42±0.04	0.36±0.06
HK activity ($nmol.min^{-1}.$(mg protein)$^{-1}$	87.5±13.0	97.1±14.2	34.4±2.9	34.2±2.7
G6PDh activity ($nmol.min^{-1}.$(mg protein)$^{-1}$	641.1±56.0	597.1±48.6	8.9±0.9	8.3±0.9
CS activity ($nmol.min^{-1}.$(mg protein)$^{-1}$	60.6±9.6	69.4±15.4	152.7±29.3	162.0±21.0
LDH activity ($\mu mol.min^{-1}.$(mg protein)$^{-1}$	118.4±25.6	104.8±15.4	164.7±37.2	162.0±31.8
GLUT activity ($nmol.min^{-1}.$(mg protein)$^{-1}$	73.1±6.5	74.1±6.8	21.0±3.8	22.0±6.3
MPO activity ($\mu mol.min^{-1}.$(mg protein)$^{-1}$	3.0±0.3	3.8±0.3	-	-
S. aureus ingestion (relative percentage)	22.1±2.0	25.2±2.1	-	-
S. aureus killed (relative percentage)	72.3±7.0	70.2±7.3	-	-

*$p<0.05$ due to control. The values are presented as mean and standard deviation of eight determinations from two experiments. Four rats were used in each experiment. (-) no determinated

DISCUSSION

Leukocytes such as neutrophils and lymphocytes use glutamine and glucose at high rates (Newsholme *et al*., 1999) to enhance cell function (Curi *et al*., 2005). Evidence is presented herein that IAA has an inhibitory effect on glucose oxidation and increases glutamine consumption by neutrophils. IAA does not alter the consumption of glucose or glutamine by lymphocytes.

Studies *in vitro* showed that the toxic effect of IAA on neutrophils, macrophages and lymphocytes is associated with cell peroxidase activity, which is high in neutrophils and absent in lymphocytes (Melo *et al*., 1998). IAA can be oxidized by peroxidase to cytotoxic species that cause lipid peroxidation and formation of strand breaks and adducts in DNA (Folkes *et al*., 1999). This study showed that IAA caused a marked increase in oxygen consumption by cultured neutrophils, an effect that was more pronounced in the presence of glutamine rather than glucose; and this auxin caused an induction in glucose and glutamine consumption by neutrophils with no alteration of lymphocytes metabolism (Melo *et al*., 1998). The metabolic alteration presented herein can be associated with the effect of IAA in presence of peroxidase.

Important enzyme activities regulate the flux of substrates through glucose and glutamine metabolic pathways; hexokinase and lactate dehydrogenase for glycolysis, glucose-6-phosphate dehydrogenase for pentose-phosphate pathway, phosphate-dependent glutaminase for glutaminolysis and citrate synthase for the Krebs cycle (Curi *et al*., 2005). Therefore, the effect of IAA on glutamine metabolism in cultured neutrophils and macrophages could well be mediated by changes in enzyme activities. Evidence is presented here, however, that the effect of IAA did not alter the enzyme activities of the glucose and glutamine pathways.

Neutrophil functions require energy (Folkes *et al*., 1999; McMurray *et al*., 1990), which is produced mainly from the metabolization of glucose to lactate (Walranda *et al*., 2003). Only 2-3% of glucose is oxidized through the Krebs cycle in neutrophils (Borregaard and Herlin, 1982). These cells also utilize glutamine at high rates, which is mainly converted to glutamate, aspartate, lactate and CO_2 (Pithon-Curi *et al*., 2003). The pentose-phosphate pathway oxidizes glucose-6-phosphate to intermediates of the glycolytic pathway, generating NADPH and ribose-5-phosphate for fatty acid and nucleotide synthesis respectively (Wood *et al*., 1963). NADPH is important for NADPH oxidase activity and for glutathione reductase to recycle oxidized glutathione in neutrophils (Pithon-Curi *et al*., 2003). Similarly, glutamine is also probably very important in neutrophils to provide glutamate for glutathione synthesis (Newsholme *et al*., 1999).

In this study, IAA administration induced glutamine oxidation by neutrophils. The increase in glutamine consumption could be a metabolic attempt to preserve the integrity and function of the cells. Studies have showed that glutamine can delay the process of apoptosis in rat and human neutrophils (Pithon-Curi *et al*., 2003). In fact, the IAA administration did not promote apoptosis or necrosis of neutrophils (Pugine *et al*., 2006). In order to confirm that IAA did not promote any alteration in cellular function, the present study showed that IAA administration do no significantly alter the parameters of microorganism engulfment/killing by neutrophils in comparison to control cells. Similarly, in the present study, the IAA administration did not show any alteration in the myeloperoxidase activity in neutrophils suggesting preservation of the cell functionality.

CONCLUSION

The observations presented led us to conclude that administration of subcutaneous indole-3-acetic acid promotes an alteration in the rat's neutrophil metabolism deduced by a decrease in glucose consumption by neutrophils and an increase in glutamine consumption; suggesting that the alteration in glucose metabolism could be compensated for by glutamine utilization by these cells. On the other hand, the metabolism alteration in rat neutrophils, promoted by IAA administration did not reduce the phagocytic capacity or myeloperoxidase activity of these cells. The effect of IAA administration, similar to *in vitro* studies, may reflect the reaction between IAA and myeloperoxidase.

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