Ouabain-induced alterations in ABCB1 of mesenteric lymph nodes and thymocytes of rats and mice

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Abstract. Ouabain is a glycoside with immunomodulating properties, and recent studies have suggested its use in adjuvant therapy for cancer treatment. Ouabain is known to modulate the immune system *in vitro,* and previous studies have revealed that ouabain can modulate the expression and activity of ABCB1, a protein associated with multidrug resistance present in immune system. Therefore, the present study investigated alterations in the expression and activity of ABCB1 in the thymi, peripheral blood monocytes and lymph nodes of Wistar rats and Swiss mice treated acutely or chronically with ouabain. A decrease of almost 45% in the monocyte count and an increase of 55% in the basophil count were observed. A significant decrease (75% reduction) in the amount of cells with ABCB1 activity was found in the thymocytes of ouabain-treated rats and mice. The possible implications of these results for cancer treatment are discussed.

Introduction

Since its discovery as a Na/K-adenosine triphosphatase (ATPase) inhibitor, ouabain has been an important topic of research in virtually all aspects of biochemistry, biophysics and physiology. A number of studies revealed the existence of an endogenous analog of ouabain in the plasma and certain mammalian tissues, including human tissues (1-3), and increased levels of this hormone are associated with hypertension (4). Furthermore, prolonged infusion of ouabain in animal models induces significant increases in blood pressure (5,6); although the mechanisms underlying the association between the onset of hypertension and enhanced levels of plasma ouabain remain unknown.

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Previously, several studies suggested an anticancer role for ouabain, which appears to be involved in complex cell-signal transduction mechanisms that result in the selective control of tumor growth (7-10). In contrast to the promising use of ouabain in tumor growth control, studies have shown that ouabain can increase the expression of ATP binding cassette subfamily B member 1 (ABCB1) (11,12). ABCB1, also known as P-glycoprotein, confers resistance to several unassociated drugs, and is therefore a major concern in cancer chemotherapy (11,13). One approach to circumvent this resistance may be to inhibit the activity of ABCB1. However, due to the important physiological actions of ABCB1 that affect several organs and tissues, including cells from the immune system, this approach is clearly not a reasonable alternative (13).

Cancer is a multifactorial disease, and cancer patients often show alterations in the immune system (14). Ouabain is known to interfere with this system (15), and ABCB1 is a protein directly associated with several functions of the immune system (16-19). At present, few studies have examined the effects of ouabain on ABCB1 expression and activity in the immune system *in vivo*. Therefore, the aim of the present study was to assess the *in vivo* effects of ouabain on immune cell counts in the blood and on ABCB1 activity in the thymus, peripheral blood mononuclear cells and mesenteric lymph nodes in order to contribute to the possible use of this glycoside in cancer therapy.

Materials and methods

Animal preparation. All animal procedures were previously reviewed and approved by the Animal Subject Committee of the UFRJ Health Science Centre (Rio de Janeiro, Brazil; protocol nos. IBCCF 082/2009 and 153/13). Male Wistar rats weighing between 260-280 g and male Swiss mice weighing between 26-32 g received food and water *ad libitum*.

Treatment with ouabain

Wistar rats. Male Wistar rats were treated with daily intraperitoneal injections of 30 μg/kg of ouabain (Sigma-Aldrich, St. Louis, MO, USA) or its vehicle, phosphate-buffered saline (PBS). A total of 20 rats were used, 12 for acute treatment (n=6 rats/group in ouabain and control groups) and 8 for chronic treatment (n=4 rats/group in ouabain and control groups). Animals were maintained under standard laboratory

conditions, with room temperature controlled (22˚C), and subjected to 12 h light-dark cycles with *ad libitum* access to food and water. Prior to the first injection at 24 h and 7 and 14 days subsequent to the injection, the rats had their blood pressure measured by a computerized tail-cuff method. The animals were sacrificed by barbiturate overdose (86 mg/kg intraperitoneal injection of pentobarbital) after 24 h (acute treatment) or 14 days (chronic treatment) of ouabain injections, and the mesenteric lymph nodes, thymi and blood were collected. Full excisions of thymi and partial excisions of mesentheric lymph nodes were performed, while blood samples were collected by caudal venous puncture prior to animals sacrifice.

Mesenteric lymph nodes and thymi were softly dissociated, and the remaining cells were washed in PBS and centrifuged at 200 x g. The pellet was suspended in ice-cold RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Heparinized blood (200 μl) was separated for an evaluation of hematological parameters, and 5 ml was centrifuged on a Ficoll-Hystopaque (Sigma-Aldrich) density gradient at 200 x g to isolate the peripheral blood mononuclear cells (PBMC). The sample was then resuspended in the same culture medium and stored on ice until required for the activity assays.

Swiss mice. A total of 8 mice were used in the present study, 4 in the control group and 4 in the ouabain-treated group. Animals were maintained under standard laboratory conditions, with room temperature controlled (22˚C), and subjected to 12 h light-dark cycles with *ad libitum* access to food and water. At 24 h subsequent to the intraperitoneal injection with 300 μg/kg of ouabain or PBS, the Swiss mice were sacrificed by barbiturate overdose (86 mg/kg intraperitoneal injection of pentobarbital). The mesenteric lymph nodes and thymi were immediately removed and softly dissociated. The remaining cells were washed in PBS and centrifuged at 200 x g. The pellet was suspended in ice-cold RPMI-1640 culture medium supplemented with 10% FBS until required for the activity assays.

Blood pressure measurement. Mean blood pressure was recorded in conscious, resting Wistar rats by a non-invasive oscillometric tail-cuff method (LE5001 Pressure Meter; Letica SA, Barcelona, Spain). One week prior to the initiation of the experiments, the rats were accustomed to restraint and inflation of the tail cuff in two independent sessions, to minimize non-specific stress. All measures were recorded in the morning, between 9 and 11 am. For each session, at least 7 blood pressure readings were recorded.

Activity assay by flow cytometry. Rhodamine (Rho)123 (Sigma-Aldrich) was used to measure ABCB1 activity in lymphocytes from PBMCs, mesenteric lymph nodes and in thymocytes, as previously described (20). The gates used to select the lymphocytes from PBMC, mesenteric lymph nodes and thymocytes were performed using forward and side scattering, according to previous descriptions (21,22).

Statistical analysis. Each experiment was performed with at least 4 animals in each group. Data are expressed as the mean ± standard error of the mean and were analyzed using two-way analysis of variance (blood pressure analyses) or an unpaired Student's *t*-test. Values of $P<0.05$ were considered to indicate a statistically significant difference.

Table I. White blood cell count of Wistar rats acutely treated with ouabain.

Cells	Control	Ouabain
Leukocytes, x$10^3/\mu$l	3.18±0.20	3.60±0.39
Basophils, %	1.55±0.23	2.41±0.23[a]
Eosinophils, %	2.34±0.26	2.25±0.32
Neutrophils, %	21.90±2.08	18.75±3.28
Lymphocytes, %	73.22±2.16	76.05±3.61
Monocytes, %	0.99±0.19	0.54±0.15[b]

[a]P=0.027, [b]P=0.048, unpaired Student's *t*-test.

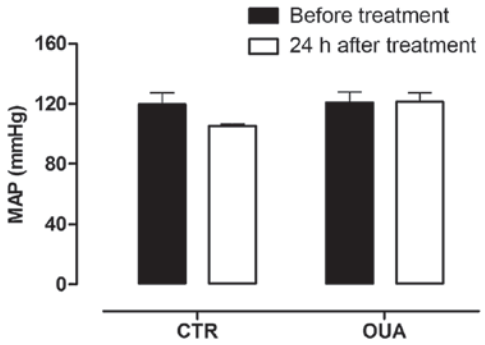

Figure 1. MAP of Wistar rats following acute treatment with ouabain. Values obtained before (full bars) and 24 h after (empty bars) the injection of 30 μg/kg of ouabain. Data are expressed as the mean ± standard error of the mean, using two-way analysis of variance (n=4). MAP, mean arterial pressure; CTR, control; OUA, ouabain.

Results

Acute ouabain treatment. As expected, the mean arterial pressure (MAP) of Wistar rats injected with a single dose of ouabain was similar to the MAP obtained prior to the administration of either ouabain or PBS (prior to injection, 121±7 mmHg; 24 h following intraperitoneal ouabain injection, 122±6 mmHg) (Fig. 1). However, significant alterations were identified in the basophil and monocyte populations, with basophil populations being increased by 55% and monocyte populations being decreased by almost 45% in blood samples from animals treated with ouabain (Table I). No alterations in red cells characteristics were observed (data not shown).

Ouabain has been shown to induce alterations in the expression and activity of ABCB1 *in vitro* (11,12). Therefore, the present study tested whether a single *in vivo* administration of ouabain could result in alterations in ABCB1 protein expression in lymph nodes and thymi. ABCB1 activity is only observed in 5-10% of thymocytes, as it is restricted to the most immature subset, the double negative cluster of differentiation (CD)4^-/$CD8^-$ subpopulation, and to the fully mature subset, the $CD4^+$ and $CD8^+$ T cells (23). Therefore, the number of cells

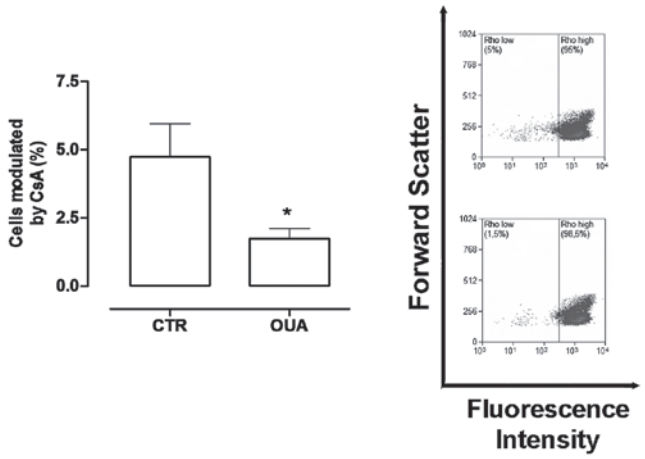

Figure 2. Modulation of ABCB1 activity in thymocytes of Wistar rats acutely treated with 30 μg/kg of ouabain. (A) Each bar represents the difference between extrusions with Rho123+CsA and Rho123 alone in the Rho low region, 24 h after the injection of ouabain. Data are expressed as the mean ± standard error of the mean, using the Student's unpaired *t*-test. *P=0.041 (n=6). (B) Representative experiment showing Rho low region, where cells poorly accumulate Rho123, indicating ABCB1 activity. Dot plots represent extrusions in the absence (upper panel) or presence (lower panel) of CsA. ABCB1, ATP binding cassette subfamily B member 1 Rho, rhodamine; CsA, cyclosporin A; CTR, control; OUA, ouabain.

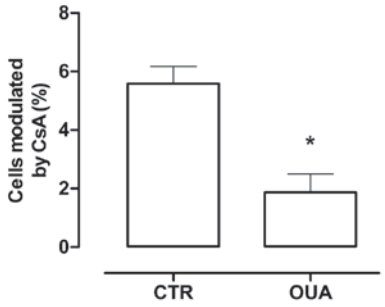

Figure 3. Modulation of ABCB1 activity in thymocytes of Swiss mice acutely treated with ouabain. Data are expressed as the mean ± standard error of the mean, using the Student's unpaired *t*-test. *P=0.028 (n=5). CsA, cyclosporin A; CTR, control; OUA, ouabain; ABCB1, ATP binding cassette subfamily B member 1.

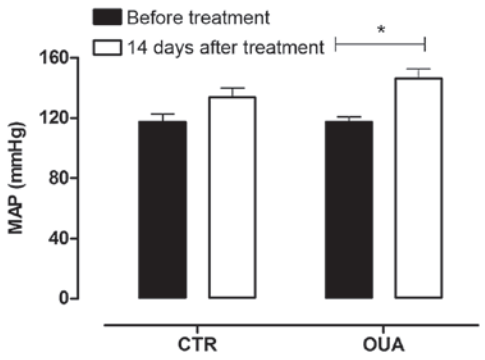

Figure 4. MAP of Wistar rats following chronic treatment with ouabain. Values obtained before (full bars) and 14 days after (empty bars) the injection of 30 μg/Kg of ouabain. Data are expressed as the mean ± standard error of the mean, using two-way analysis of variance. *P=0.004 (n=4). MAP, mean arterial pressure; CTR, control; OUA, ouabain.

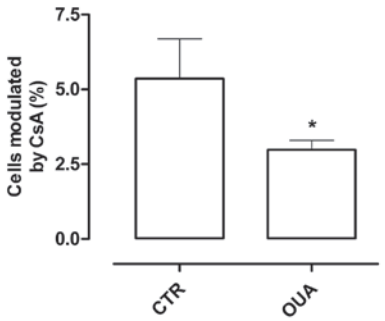

Figure 5. Modulation of ABCB1 activity in thymocytes of Wistar rats chronically treated with ouabain. Data are expressed as the mean ± standard error of the mean, using the Student's unpaired *t*-test. *P<0.05 (n=4). CsA, cyclosporin A; CTR, control; OUA, ouabain; ABCB1, ATP binding cassette subfamily B member 1.

presenting ABCB1 activity in the thymus was determined by the percentage of cells with low Rho content, which were modulated by the ABCB1 inhibitor cyclosporin A (CsA) (Fig. 2A). However, as the majority of mature lymphocytes demonstrate ABCB1 activity, the gates used to analyze the percentage of cells modulated by CsA were drawn by dividing the population into two halves, using the incubation with Rho123 as a reference (Fig. 2B).

Fig. 2A shows that the percentage of thymocytes with ABCB1 activity in control rats was almost 5%, which was obtained by corroborating previous results (23); however, in the thymocytes of rats pretreated with ouabain, this activity decreased by ~50% of the control. Treatment with ouabain did not alter the number of cells modulated with CSA in PBMCs or mesenteric lymph nodes (data not shown).

To evaluate whether the observed effect in thymocytes was species dependent, the same experiments were performed in mice. Due to the difficulties in obtaining and separating blood cells from the mice, only lymph nodes and thymi were tested. As shown in Fig. 3, the same results were obtained, with an approximate 50% reduction of ABCB1 activity in the thymi and no difference in lymph nodes.

Chronic ouabain treatment. Since a chronic treatment with ouabain may lead to hypertension and, to the best of our knowledge, no studies assess ABCB1 activity in chronic ouabain-treated animals, the present study also tested the effects of chronic ouabain treatment on ABCB1 activity in the lymph nodes and thymi of rats. The rats were treated daily with intraperitoneal injections of 30 mg/kg ouabain. The MAP was measured on days 0, 7 and 14. As shown in Fig. 4, after 14 days the MAP of ouabain-treated rats was significantly elevated. The animals were then sacrificed and the ABCB1 activity was measured. Fig. 5 shows that the effect observed in the thymus with acute ouabain treatment remained present following chronic treatment. In addition, the ABCB1 activity of mesenteric lymph nodes was also diminished after chronic treatment with ouabain.

Discussion

To the best of our knowledge, the present study is the first to evaluate the effects of acute ouabain treatment in the blood. The

dose of ouabain used (30 μg/kg) has been previously shown to induce hypertension when infused or released subcutaneously for long periods of time in Wistar rats (6,24). Corroborating with the results of other studies (6,24), the current study found that ouabain increased the MAP after 14 days of continuous daily treatment, but did not promote any alteration in the blood pressure of Wistar rats after 24 h. However, the acute administration of ouabain was found to induce significant alterations to the basophil and monocyte counts in the peripheral blood.

Basophils are granulocytes known to participate in allergic reactions and parasite infections. This type of cell expresses major histocompatibility complex class II, CD80 and CD86, and has the potential to capture and process antigens and present peptides to effector cells, which promote the development of T helper 2 cells via early secretion of interleukin (IL)-4 during allergic immune responses (25). The differentiation of bone marrow precursors in basophils requires IL-3, and these differentiated cells are capable of producing histamine, IL-4, IL-13 and leukotriene C4 (26), which is an important substrate described for another member of the ABC superfamily, ABCC1 (27). Basophilia has a strong association with certain malignancies, particularly acute and chronic myeloid leukemia (26).

Monocytes are mononuclear cells present in the blood, which, after activation, migrate from the blood to the tissues and usually differentiate into macrophages. Monocytes are important for the initiation, amplification and shutdown of the immune response through the production of cytokines, recognition of pathogen-associated molecular patterns, antigen presentation and phagocytosis (28-30). Ebner *et al* (31) demonstrated that monocytes can differentiate in dendritic cells after stimulation with IL-3 and IL-4.

The actions of ouabain are extremely diverse and may vary according to the target organ or tissue. Certain studies have shown important implications of the ouabain hormone in cell proliferation, muscle contraction and cell survival in distinct cell types (32-34). In the immune system, ouabain modulates the expression of several important receptors in lymphocytes and monocytes (35,36), impairs lymphocyte proliferation induced by mitogens (36) and induces cytokine secretion in PBMC (37,38). In addition, the *in vivo* administration of ouabain has been shown to reduce the amount of mature B lymphocytes in the spleen and prevent the inflammation induced by several substances in mice (39,40).

Although no studies make direct associations between ouabain and IL-3, certain studies have shown that ouabain interferes with other interleukin pathways (41,42) and that IL-3 activates Na^+/K^+-ATPase, which is the main target of ouabain, in macrophages (43). Therefore, the possibility that ouabain regulates the synthesis or secretion of IL-3, which could result in augmented number of basophils and reduced number of monocytes in peripheral circulation, cannot be ruled out. In addition, it has been reported that ouabain affects the activation of monocytes and modulates their functions, acting as an immunomodulator of these cells (44).

At present, the consequences of the observed alterations in leukocyte counts that were induced by short-term treatment with ouabain remain unclear; however, these alterations could potentially affect the immune response of cancer patients. Since basophilia has been shown to be associated with a bad prognosis (reduced survival) in myelodysplastic syndromes (45) and also in patients with solid tumors (46), the present observations suggest that additional studies are required to assess the use of ouabain for treatment in cancer patients.

Ouabain is known to modulate the expression of two ATPases associated with cancer resistance, ABCB1 and ABCC1 (47). Therefore, the present study aimed to assess whether ouabain could alter the expression or activity of ABCB1 in PBMC and thymocytes *in vivo*. Indeed, acute and chronically ouabain-treated Wistar rats displayed significant decrease in ABCB1 activity in lymphocytes from the thymus. Despite being more resistant to ouabain compared with rats (48), which explains the greater dose (300 μg/kg) used, Swiss mice acutely treated with the ABCB1 glycoside also presented similar results, indicating that this effect is not species-dependent.

ABCB1 is associated with the transport of several substrates, including lipids, hormones and cytokines (19,49,50). In thymocytes, the expression of this protein is restricted to the double negative ($CD4^-/CD8^-$) thymocytes and to the simple positive populations $CD4^+$ and $CD8^+$, representing the most immature subset and the mature thymocyte populations, respectively (23). Thus, the observation that ouabain induced a significant decrease in the percentage of thymocytes with ABCB1 activity raises at least three hypotheses: i) That thymocytes with ABCB1 are simply undergoing protein downregulation in the plasma membrane; ii) that thymocyte maturation is being impaired, leading to an accumulation of double positive cells ($CD4^+/CD8^+$), which do not exhibit ABCB1 activity; or iii) that the thymocytes with decreased ABCB1 activity after the ouabain injection could be undergoing apoptosis. Although, at present, the significance of the present observations is not understood, the findings suggest that ouabain could contribute to immunodeficiencies in cancer patients, which argues against its use in cancer treatment.

As the immune system is directly associated with the development of cancer, and since acute and chronic treatment with ouabain were able to alter the activity of ABCB1 in immature lymphocytes and the percentage of monocytes and basophils in the peripheral blood, the current results suggest that additional studies are required to assess the use of ouabain as an adjuvant anticancer therapy, and may also contribute to elucidating the mechanisms of development of hypertension triggered by ouabain.

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