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Effects of methotrexate on calcium flux in rat liver mitochondria, microsomes and plasma membrane vesicles

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Abstract

The metabolic effects of methotrexate in perfused livers are similar to those exerted by hormones acting through Ca^{2+}-dependent mechanisms. The aim of the present study was to determine whether the effects of methotrexate are mediated by a direct action on cellular Ca^{2+} fluxes. Methotrexate did not affect the ATP-dependent $^{45}Ca^{2+}$ uptake by mitochondria, microsomes and inside-out plasma membrane vesicles and Ca^{2+} efflux from plasma membrane vesicles. However, methotrexate was able to stimulate $^{45}Ca^{2+}$ release from preloaded microsomes. The amount of Ca^{2+} released by methotrexate was similar to that induced by IP_3. Methotrexate could be acting through the capacitative calcium entry mechanism.

Keywords: Calcium transport; Liver; Methotrexate; Microsomes; Mitochondria; Plasma membrane

1. Introduction

Methotrexate (MTX) is a folate antagonist widely used in the treatment of neoplastic diseases. It has also been used successfully as anti-inflammatory and immunosupressive agent in non-neoplastic diseases such as psoriasis, arthritis, biliary cirrhosis and Reiter's syndrome (Weinblatt, 1985; Cronstein et al., 1991; Genestier et al., 2000). Methotrexate is actively accumulated in the liver where it is metabolized and stored in polyglutamated form. The major side-effect of chronic methotrexate administration is hepatotoxicity, which is characterized by fatty infiltration, inflammation, cellular necrosis and apoptosis, steatosis, fibrosis and cirrhosis (Barak et al., 1985; Kobayashi et al., 2002).

Previous studies performed in our laboratory have demonstrated that methotrexate affects liver metabolic pathways modulated by Ca^{2+} ions. It stimulates hepatic glycogenolysis (Oliveira et al., 1986; Yamamoto et al., 1992), inhibits ketogenesis from long-chain fatty acids (Nishiyama et al., 1996a) and activates gluconeogenesis from alanine (Nishiyama et al., 1996b). Methotrexate presents some features that are similar to those observed for Ca^{2+}-mobilizing agents such as norepinephrine, phenylephrine, angiotensin II and vasopressin (Yamamoto et al., 1992): it is inactive on glycogenolysis when the livers are depleted of intracellular Ca^{2+} and perfused with Ca^{2+}-free medium, but subsequent addition of extracellular Ca^{2+} causes restoration of glycogenolytic action; when intracellular stores are not depleted but Ca^{2+} is absent from the extracellular medium, methotrexate produces only transient increases in glycogenolysis. Thus, like Ca^{2+} mobilizing hormones, both intracellular and extracellular Ca^{2+} are required to induce and to sustain the metabolic responses to methotrexate. It was also demonstrated that methotrexate produces transient increases in $^{45}Ca^{2+}$ efflux in perfused livers preloaded with $^{45}Ca^{2+}$ and perfused with Ca^{2+}-free medium (Yamamoto et al., 1992). In spite of these similarities, specific blockers of norepinephrine, phenylephrine, angiotensin II, vasopressin and isoproterenol are unable to inhibit the action of methotrexate in the perfused rat liver (Yamamoto et al., 1992). Collectively, these observations lead to the hypothesis that methotrexate can act intracellularly without participation of receptors or secondary messengers. It can be postulated that methotrexate mimics the action of Ca^{2+}-dependent hormones by elevating itself the

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cytosolic Ca^{2+} levels. Methotrexate could be acting, for example, on Ca^{2+} release from intracellular stores, including those ones of mitochondria or endoplasmic reticulum, or on Ca^{2+} fluxes through the plasma membrane. In intact cell systems, such as the perfused liver (Yamamoto et al., 1992) or isolated hepatocytes, it is almost impossible to distinguish all these possibilities. For this reason, the approach adopted in this study was to perform a comparative study of the effects of methotrexate on Ca^{2+} fluxes in isolated mitochondria and in vesicles from endoplasmic reticulum and plasma membranes. To allow effective comparisons, methotrexate was investigated at a concentration that was shown to be active in the isolated perfused rat liver, i.e., 0.4 mM (Yamamoto et al., 1992). It should be mentioned that this concentration is achieved in the plasma of adult patients following chemotherapeutic doses of methotrexate (Bore et al., 1987; Vakily et al., 2005).

2. Material and methods

2.1. Reagents

Sodium methotrexate (*N*-[4(2,4-diamino-6-pteridinylmethylamino)benzoyl]-L-glutamic acid) was purchased from Lederle Parenterals (Carolina, Puerto Rico, USA). AMP (adenosine 5′-monophosphate), ATP (adenosine 5′-trisphosphate), IP_3 (inositol 1,4,5-trisphosphate), glucose 6-phosphate, mannose 6-phosphate, Na_3VO_4 (sodium orthovanadate), succinate, PMSF (phenylmethyl-sulfonylfluoride), hexokinase, oligomycin, ruthenium red and Percoll were purchased from Sigma Chemical Co. (St. Louis, MO, USA). $^{45}CaCl_2$ was purchased from NEN Life Science Products (Boston, MA, USA) and the glucose-oxidase kit was from Labtest Diagnóstica (São Paulo, Brazil). All other reagents were the best available grade. Methotrexate was dissolved in distilled water. Oligomycin was solubilized in ethanol. Controls were performed to exclude solvent effects, but no significant changes were found.

2.2. Preparation of mitochondria

Liver mitochondria were isolated from male Wistar rats (*Rattus norvegicus*) weighing 220 to 250 g and fed ad libitum. Livers were homogenized in the extraction medium described below, using a loose-fitting Dounce homogenizer. The homogenate was fractionated by sequential centrifugations at 536 and 7080 ×*g*, for 10 min. After two wash cycles by suspension and centrifugation at 6392 ×*g*, the final mitochondrial pellet was suspended in a small volume of medium to give a protein concentration of 40–50 mg/mL. All operations were performed at 0–4 °C. The extraction medium contained: 0.25 M sucrose, 0.01 M Mops, pH 7.4, and 0.1 mM phenylmethylsulphonylfluoride (PMSF). The same medium was used for suspending the final mitochondrial pellet.

2.3. Preparation of microsomes

Microsomes were isolated from livers through a combination of procedures described elsewhere (Mihara and Sato, 1972; Moore et al., 1975; Bygrave, 1978; Schanne and Moore, 1986). Some modifications were done to obtain microsomal vesicles with minimal contamination with other membrane vesicles. Liver microsomal fractions were prepared from male Wistar rats (220–250 g), deprived of food overnight and killed by decapitation. The liver was removed, sliced and homogenized in a medium containing 1.15% KCl, 0.02 M Tris, pH 7.4, and 0.1 mM PMSF, using a Dounce homogenizer. The homogenate was filtered through four layers of gauze and centrifuged at 2550 ×*g* for 10 min. The supernatant was sequentially centrifuged at 7080 ×*g* (10 min), at 12,434 ×*g* (10 min) and finally at 105,000 ×*g* (60 min). The resulting pellet was suspended in the same medium (≅35 mg protein/mL). All procedures were conducted at 0–2 °C. The microsomes were either used on the same day or stored in liquid nitrogen. To evaluate the purity and the enrichment of the microsomal vesicle preparations, the activities of the plasma membrane marker enzyme 5′-nucleotidase, the endoplasmic reticulum marker enzyme glucose 6-phosphatase and the total $Ca^{2+}Mg^{2+}$-ATPase were assayed. The microsomal fractions showed high purity as indicated by the glucose 6-phosphatase/5′-nucleotidase ratio (11.62±0.85, $n=10$). Measurements of the $Ca^{2+}Mg^{2+}$-ATPase activity revealed that the contamination by mitochondria was also minimal. When microsomes were incubated in the presence of 12.5 μg/mL oligomycin, only a small inhibition was found in the $Ca^{2+}Mg^{2+}$-ATPase activity (−20%; $p=0.147$), in contrast to that observed with the crude homogenate incubations (−73%; $p<0.0001$). The test of accessibility for mannose 6-phosphate revealed that microsomes also presented high degree of integrity in crude liver homogenates and purified fractions. The hydrolytic activities of the glucose 6-phosphatase multicomponent system when 1.0 mM glucose 6-phosphate and 1.0 mM mannose 6-phosphate were used as substrates were, respectively, 293.0±16.9 ($n=7$) and 29.75±3.0 ($n=11$) nmol phosphate min^{-1} mg $protein^{-1}$.

2.4. Preparation of rat liver plasma membrane vesicles

Liver plasma membrane vesicles were isolated essentially as described by Armstrong and Newman (1985). Several modifications were introduced to adapt the procedures to our experimental conditions and to increase purity and recovery. Livers were removed from ad libitum fed male Wistar rats (220–250 g). The liver was rinsed and homogenized in cold medium containing 0.25 M sucrose, 0.02 M Tris, pH 7.4 and 0.1 mM PMSF (S–T buffer) in a Dounce homogenizer. The homogenate was filtered through gauze, diluted to 10% in the same medium and centrifuged at 1434 ×*g* for 10 min. Aliquots of 17 mL of the supernatant were layered on 10 mL of 1.45 M sucrose plus 0.025 M Tris, pH 7.4, medium and centrifuged at 35,000 ×*g* for 30 min. After vacuum aspiration of the upper phases, the plasma membrane fraction (a pale tan interfacial layer) was displaced upward by the addition of 5–6 mL of 1.35 M sucrose plus 0.025 M Tris medium (pH 7.4) underneath the membrane layer. The membrane pellet was collected and diluted four-fold in isotonic S–T buffer and centrifuged for 15 min at 10,000 ×*g*. After vacuum aspiration of the