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Interaction of piroxicam with mitochondrial membrane and cytochrome *c*

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Abstract

Modulation of surface properties of biomembranes by any ligand leading to permeabilization, fusion, rupture, etc. is a fundamental requirement for many biological processes. In this work, we present the interaction of piroxicam, a long acting Non-Steroidal Anti-Inflammatory Drug (NSAID) with isolated mitochondria, membrane mimetic systems, intact cells and a mitochondrial protein cytochrome *c*. Dye permeabilization study on isolated mitochondria indicates that piroxicam can permeabilize mitochondrial membrane. Direct imaging by Scanning Electron Microscope (SEM) shows that piroxicam induces changes in mitochondrial membrane morphology leading to fusion and rupture. Transmission Electron Microscope (TEM) imaging of piroxicam treated DMPC vesicles and mixed micelles formed from CTAB and SDS show that causing membrane fusion is a general property of piroxicam at physiological pH. In intact cells viz., V79 Chinese Hamster lung fibroblast, piroxicam is capable of releasing cytochrome *c* from mitochondria into the cytosol in a dose dependent manner along with the enhancement of downstream proapoptotic event viz., increase in caspase-3 activity. We have also shown that piroxicam can reduce cytochrome *c* within a time frame relevant to its lifetime in blood plasma. UV-visible spectroscopy has been used to study the reaction mechanism and kinetics in detail, allowing us to propose and validate a Michaelis–Menten like reaction scheme. CD spectroscopy shows that small but significant changes occur in the structure of cytochrome *c* when reduced by piroxicam.

Keywords: NSAIDs; Mitochondria; Membrane fusion; Cytochrome *c*; Kinetics; Optical spectroscopy

1. Introduction

Modification of the surface properties of membranes by any ligand could lead to several phenomena like aggregation, leakage of trapped contents or permeabilization, fusion, etc. Such modulation of surface properties is a fundamental requirement for many biological processes [1]. Piroxicam [4-hydroxy-2-methyl-*N*-(pyridin-2-yl) -2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], a drug belonging to the oxicam group of NSAIDs, is not only a good anti-inflammatory agent, but also show chemopreventive and chemosuppressive effects in different cancer cell lines and animal models [2–4]. The molecular mechanism behind its anti cancer effect is not yet fully understood. Piroxicam strongly interacts with membrane mimetic systems like micelles and vesicles [5–8]. Different membrane parameters viz. electrostatics and hydrophobicity, affect the transformation or switchover between different prototropic forms of piroxicam in such a way so as to exert a profound effect in dictating which structural form will predominate in solution and would be incorporated in the membrane mimetic systems. To extend our study to biomembranes we chose mitochondrial membrane. Mitochondria are in general a very good pharmacological target [9,10]. It is known that several drugs

Abbreviations: NSAID, Nonsteroidal anti-inflammatory drug; SEM, Scanning electron microscope; TEM, Transmission electron microscope; DMPC, Dimyristoyl phosphatidylcoline; COX, Cyclooxygenase; MOPS, 3-Morpholinopropane sulphonic acid; DMSO, Dimethyl sulfoxide; PBS, Phosphate buffer saline; EGTA, Ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, Ethylenediaminetetraaceticacid; PTA, Phosphotungstic acid; CD, Circular dichroism; PMSF, Phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene difluoride

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including NSAIDs can affect the mitochondrial membrane potential, respiration, energy coupling and can also lead to outer membrane permeabilization [9]. Mitochondrial outer membrane permeabilization results in release of proteins like cytochrome *c*, normally found in the space between the inner and outer mitochondrial membranes. Cytochrome *c* release in the cytosol causes caspase activation that orchestrates the downstream events associated with apoptosis [11,12]. Hence, affecting mitochondrial membrane by drugs is an important strategy in chemotherapy [11]. Conventional chemotherapeutic agents result in mitochondrial permeabilization through an indirect way, via pro-apoptotic second messengers, whose nature depends on the apoptosis inducing agent. Such secondary messengers, for example BH3-only members of the Bcl-2 protein family and other proteins, activate the pro-apoptotic BH123 proteins, Bax and Bak, to oligomerize and insert into the outer mitochondrial membrane leading to mitochondrial outer membrane permeabilization [11,12]. This initiates the down-stream cascade of pro-apoptotic events. COX-2 inhibitors like NS-398 and celecoxib have been shown to induce apoptosis mediated by the release of cytochrome *c* from mitochondria with the consequent activation of downstream caspases like caspase-3, caspase-8 and caspase-9, etc. [13,14]. Several different reasons for the release of cytochrome *c* by these COX-2 inhibitors have been proposed, of which modulation of mitochondrial morphology is a possibility.

In this study we are interested to see if piroxicam, a COX-inhibitor, can exert its effect on mitochondrial membrane morphology, thereby leading to the mitochondrial permeabilization. To do so, we have carried out the mitochondrial permeabilization study with cell free isolated mitochondria in presence of piroxicam only. The modulation of mitochondrial membrane morphology by piroxicam has also been directly imaged by Scanning Electron Microscope. To see whether the effect of piroxicam on mitochondrial membrane can have any consequence on cellular processes, we have extended our studies to intact cells. Finally, we show that piroxicam can directly interact with the mitochondrial protein cytochrome *c* in vitro, within a time frame relevant to its lifetime in blood plasma. UV-Visible absorption and CD-spectroscopy have been used to study the slow kinetics of interaction of piroxicam with cytochrome *c*. We are aware that at the cellular level, the effect of piroxicam can also occur via several other parallel pathways e.g., by inducing secondary effectors involved in the physiological control of apoptosis, etc.; but the fusion event observed in the membrane mimetic systems implies that there is a very strong physical interaction of piroxicam with mitochondrial membrane. So, in this study, we only aim to demonstrate the effect of piroxicam at four levels, on isolated mitochondrial membrane, on membrane mimetic systems, on cellular mitochondria and on the mitochondrial protein cytochrome *c*. Identification of pathways other than modulation of mitochondrial morphology for the release of cytochrome *c* and caspase activation in intact cells is the subject of a separate study.

2. Experimental procedures

2.1. Reagents

Piroxicam, DMPC and MOPS were purchased from Sigma Chemicals (US) and were used without further purification. Stock solution of piroxicam of concentration 3 mM was prepared in DMSO (Merck, Germany) and the exact concentration was adjusted by corresponding buffer. Mitotracker Green FM® was purchased from Molecular Probes, USA. Cytochrome *c* and Glycine were purchased from SISCO Research Laboratory (SRL), India.

2.2. Cell culture and treatment

Chinese hamster lung fibroblast V79 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum (Sigma) and antibiotics (penicillin and streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere in plastic petridishes. Cells were grown to confluency and fresh medium was added along with 20, 40 100 μM piroxicam. Equivalent amount of DMSO was added to the control cells to compensate the DMSO present in solution when piroxicam is diluted from stock DMSO solution.

2.3. Immunoblot analysis

After treatment with 1 μM staurosporine (Almone Laboratories, Israel), piroxicam at concentration of 20, 40 and 100 μM respectively, cells were washed in PBS buffer (pH 7.4) and suspended in lysis buffer (20 mM Tris, 1 mM EGTA, 1 mM EDTA, 5 mM NaF and a cocktail of protease inhibitors of 10 μM pepstatin A, 10 μM leupeptin, and 1 mM PMSF, pH 7.5). Cells were ruptured by rapid freeze–thaw and homogenized on ice. Lysed cells and nuclei were pelleted by centrifugation at 1000×*g* for 10 min. The supernatant was centrifuged further at 15000×*g* for 40 min at 4 °C. The resultant supernatant was designated as the cytosolic fraction, which was used for the detection of cytochrome *c*. The supernatant was taken for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein content was quantified according to Bradford [15] and then solubilized in Laemmli sample buffer. The samples were boiled for 5 min at 100 °C and loaded onto a 15% acrylamide gel. Electrophoresis was carried out at a constant voltage of 130 V. Cellular proteins were transferred electrophoretically to a PVDF membrane using an Atto Electroblotter apparatus. The transfer buffer (pH 8.3) contained 96 mM glycine, 10 mM Tris, and 10% methanol. The transfer was carried out for 50 min at constant current of 160 mA. Hydrophobic or nonspecific sites were blocked overnight at 4 °C with 5% BSA in Tris-buffered saline (50 mM Tris and 150 mM NaCl) containing 0.1% Tween 20 (TBS-T). Membranes were washed four times for 15 min in TBS-T. The blots were probed with the primary antibody anti-cytochrome *c* (Cell Signaling Technology, USA) and anti-β-actin (Sigma) in TBS-T, 1% bovine serum albumin for 2 h at room temperature. Membranes were washed four times for 15 min and incubated for 1 h at room temperature with alkaline phosphatase (ALP)-conjugated secondary antibody (1:1000) in TBS-T containing 1% BSA (pH 7.5). Secondary antibodies consisted of ALP-conjugated anti-rabbit IgG (Oncogene Research, USA) for cytochrome *c* and ALP-conjugated anti-mouse IgG for β-actin (Oncogene Research, USA). PVDF membranes were washed four times for 15 min, and cytochrome *c* and β-actin were detected colorimetrically with NBT-BCIP as substrate. Western blots were scanned by a UMAX Astra Scanner and the bands were quantified by using Scion Image Beta 4.02 software from Scion Corporation, USA.

2.4. Isolation of mitochondria

Cells were washed in PBS and suspended in lysis buffer. Cells were ruptured by rapid freeze–thaw and homogenized on ice. Lysed cells and nuclei were pelleted by centrifugation at 1000×*g* for 10 min. The supernatant was centrifuged further at 15,000×*g* for 40 min at 4 °C. Pellet of Mitochondria was collected.

2.5. Measurement of caspase-3 activity

Caspase-3 assay was done by the caspase-3 fluorescence assay kit from BD-Pharmingen, following the protocol mentioned in the kit. Caspase-3 activity is given in arbitrary fluorescence units.