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EFFECT OF 5-NITROINDOLE ON ADENYLATE ENERGY CHARGE, OXIDATIVE PHOSPHORYLATION, AND LIPID PEROXIDATION IN RAT HEPATOCYTES

MARTA DUBIN,* PATRICIA H. CARRIZO,† ANA M. BISCARDI, SILVIA H. FERNANDEZ VILLAMIL and ANDRÉS O. M. STOPPANI*‡

Centro de Investigaciones Bioenergéticas, Facultad de Medicina, Paraguay 2155, (1121)-Buenos Aires, Argentina

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Abstract—5-Nitroindole (NI), a mutagenic nitroarene, was assayed for cytotoxic effects on rat hepatocytes. After incubation with 25–100 μM NI, the adenylate energy charge of the hepatocytes decreased significantly as a result of the decrease in ATP and the increase in AMP. ATP depletion correlated well with the effects of NI on mitochondrial electron transfer and energy transduction in hepatocytes. Thus, NI (a) inhibited the antimycin-sensitive hepatocyte respiration; (b) inhibited NADH oxidation by disrupted hepatocyte mitochondria; (c) inhibited L-malate-L-glutamate oxidation by ADP-supplemented mitochondria; (d) in the absence of ADP, stimulated the same substrates and also succinate oxidation by mitochondria; (e) released the latent ATPase activity of mitochondrial F_1F_0-ATP synthase; (f) shifted the redox level of reduced cytochromes ($c + c_1$) and b towards the oxidized state; (g) inhibited NADH oxidation by disrupted mitochondria in the vicinity of the NADH-dehydrogenase flavoprotein; (h) inhibited Ca^{2+} uptake by mitochondria using L-malate-L-glutamate as an energy source; (i) inhibited valinomycin-induced, endogenously energized K^+ uptake, with little effect on the ATP-induced uptake; and (j) inhibited the MgATP-dependent contraction of Ca^{2+}-swollen mitochondria. NI inhibited lipid peroxidation in hepatocytes and also in substrate-supplemented liver microsomes and mitochondria, thus ruling out hydroperoxides as a cause of NI cytotoxicity. Long-term incubation with NI produced loss of hepatocyte viability, as indicated by lactate dehydrogenase leakage.

Key words: 5-nitroindole; nitrofuran compounds; hepatocytes; adenylate energy charge; oxidative phosphorylation; lipid peroxidation

Heterocyclic nitroarenes are widely used as chemotherapeutic agents. In a series of nitroarenes tested for their mutagenic activity on *Salmonella*, NI§ proved to be one of the most effective [1–4]. Since no information was available on the action of this compound on eukaryotic cells, it seemed of interest to assay NI in rat hepatocytes, a procedure that constitutes a powerful tool in mechanistic studies on the metabolic action of xenobiotics [5]. Cytotoxicity was investigated using several biochemical parameters, namely (a) the ATP level in hepatocytes [6], (b) the adenylate energy charge [7], (c) electron transfer; (d) lipid peroxidation [8–10], and (e) release of LDH [11]. Moreover, the following parameters were investigated in isolated mitochondria: (a) electron transfer and energy coupling; (b) Ca^{2+} and K^+ uptake, and (c) F_1F_0-ATP synthase ATPase activity. The adenylate energy charge [the ratio (ATP + 0.5 ADP)/(ATP + ADP + AMP)] [7] depends essentially on the intracellular ATP level, a limiting factor for cell viability [6]. Lipid peroxidation is one of the basic mechanisms of cell damage [8–10], and alteration of mitochondrial permeability by lipid peroxidation products may cause ATP depletion [10]. Finally, irreversible injury renders the hepatocyte plasma membrane unable to prevent the leakage of cytosolic molecules, particularly protein molecules, thus contributing to cell death [6, 12].

Studies on structure–activity relationships indicate that the nitro group may be responsible for the biological activity of nitro compounds [13–16]. Redox-cycling reactions of these compounds may produce oxygen radicals that are responsible for their toxicity [16–18]. Accordingly, the effects of NI were compared with those of nitrofuran compounds characterized by their capability of producing oxygen radicals, such as the antichagasic compound nifurtimox [16–18].

MATERIALS AND METHODS

Animals. Male Wistar rats (220–250 g) were used in the experiments. Animals were fed a Purina-like

* Career Investigator, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

† Research Fellow, Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

‡ Corresponding author. Tel. and FAX: 54-1-961-6521.

§ Abbreviations and chemical terms: NI, 5-nitroindole; LDH, lactate dehydrogenase; DMFA, *N*,*N*-dimethylformamide; TCA, trichloroacetic acid; MGM, L-malate-L-glutamate-malonate; *t*-BuOOH, *tert*-butylhydroperoxide; TBA, thiobarbituric acid; MDA, malondialdehyde; G6P, glucose-6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; NF, (5-nitrofurfurylidene)amino; nifurtimox, tetrahydro-3-methyl-4-[NF]-2*H*-1,4-thiazine-1,1-dioxide; NF-pyrazole, 1-[NF]-pyrazole; NF-triazole, 4-[NF]-1,2,4-triazole; and DETAPAC, diethylenetriaminepentaacetic acid.

rat chow. The protein content of the diet was 23% and included all the essential amino acids.

Chemicals. Collagenase A from *Clostridium histolyticum* was purchased from Boehringer Mannheim GmbH, Germany. D-Glucose, sucrose, NADH, ATP, ADP, AMP, CTP, potassium phospho(enol)pyruvate, pyruvate kinase, adenylate kinase, luciferase-luciferin, *t*-BuOOH, DMFA, HEPES, bovine serum albumin (A-4503), L-malic acid, L-glutamic acid, malonic acid, succinic acid, sodium pyruvate, sodium ascorbate and Trypan Blue were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. Other reagents were obtained from the suppliers indicated [18–20] and were of the highest purity available.

Isolation of hepatocytes. Hepatocytes were isolated from the livers of fed rats that were anesthetized i.p. with 20 μmol sodium pentobarbital (5 mg/100 g body weight). The liver cells were isolated by the method of Seglen [21]. Briefly, after catheterization of the portal vein, the liver was perfused *in situ*, for 10 min, at a rate of 40 mL/min, with a Ca^{2+}-free medium [medium A (mM): HEPES, 10; NaCl, 135; KCl, 6.7 and glucose, 5; pH 7.4] saturated with O_2 and maintained at 37°. Then the liver was excised and perfused with 120 mL of medium A to which $CaCl_2 \cdot 2H_2O$ (5 mM) and collagenase (50 mg/100 mL) had been added (medium B). The collagenase solution had been filtered previously through a Millipore 0.45 μm filter. Perfusion was performed using a recirculating system, at 37°. If needed, the medium pH was brought to pH 7.4 by adding 100 μL of 1 N NaOH, 4 min after the beginning of recirculation. After 10 min of perfusion, medium B was replaced by medium A for 2 min, in order to exclude the remaining collagenase and cell debris. The liver was transferred to a beaker filled with 30 mL of medium B. The cells were dispersed rapidly with a stainless-steel comb, and vascular and connective tissues were discarded. After filtration on Nytrel TI 500 (Ets Desjobert, Paris, France), the suspension was incubated for 4 min at 37° and filtered on nylon (150 μm, Filtration Armentières, Paris, France). The suspension was diluted with 120 mL of medium C containing (mM): NaCl, 130; KCl, 5.2; $MgSO_4$, 0.9; $CaCl_2 \cdot 2H_2O$, 0.12; Tris–HCl, 20 and Na_2HPO_4-KH_2PO_4, 3.0; pH 7.4, saturated with O_2, and centrifuged at 50 *g* for 90 sec. The cells were resuspended in 50 mL of medium C, allowed to settle for 10 min at 0°, and resuspended in medium C, to make a suspension of approximately 5×10^6 hepatocytes per mL. The hepatocyte suspension was filtered through nylon (60 μm) and placed in culture flasks (25 cm^2 area; Falcon, Division of Becton, Dickinson & Co., Oxnard, CA, U.S.A.), on ice. Cells were counted under the microscope with a hemocytometer, and viability was estimated by the Trypan Blue exclusion method or the LDH leakage method. Hepatocytes showing an initial viability of greater than 85%, measured by the Trypan Blue exclusion method [12], were used for the experiments, for no more than 4 hr after isolation.

Incubation of hepatocytes. Hepatocyte suspensions were diluted with Krebs–Henseleit buffer–5.0 mM glucose solution (pH 7.4) [8] to a concentration of about 3×10^6 cells/mL. Duplicate samples, containing 3×10^6 cells/mL, were incubated in Erlenmeyer flasks, with additions as indicated under Results. Incubations were performed in a New Brunswick Gyrotory Water Bath Shaker, model G76, at 37° and at a rate of 90 strokes/min, under 95% O_2 + 5% CO_2 atmosphere. NI was added at zero-time, dissolved in DMFA. Controls received the same volume of solvent ("DMFA-samples") whose concentration never exceeded 1% (v/v). Aliquots of incubation mixtures were taken as indicated under Results, for measuring cellular biochemical parameters.

Mitochondrial and microsomal preparations. Mitochondria were prepared from starved rats as described [22], using 0.24 M sucrose, 1.0 mM EDTA, 50 mM Tris–HCl buffer, pH 7.4, as homogenization medium. After two washings in the centrifuge at 6800 *g* with the homogenization medium, the mitochondria were suspended in the same medium at a concentration of about 25 mg protein/mL. When necessary, mitochondria were disrupted by freezing–thawing three times in liquid nitrogen. Microsomal preparations were obtained as described [19, 20].

Hepatocyte viability. Leakage of cytosolic LDH was assayed as described [12]. Briefly, at the times indicated under Results, preincubated hepatocytes were allowed to settle in an ice-bath, and 50 μL samples of supernatant were carefully taken, without disturbing the cell layer. LDH activity in these samples was measured spectrophotometrically, at 340 nm, using an assay mixture containing 50 μL of supernatant, 0.2 mM NADH, 1.05 mM sodium pyruvate and Krebs–Henseleit medium (pH 7.4), containing 2% (w/v) bovine serum albumin; total volume, 3.0 mL [12].

Adenine nucleotide assay. Levels of adenine nucleotides in hepatocytes were measured by the luciferase–luciferin method [23, 24], using a Packard Pico-Lite Luminometer. Samples (0.2 mL) of the incubation mixture (6.0×10^5 hepatocytes) were centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was discarded, and the pellet was frozen and thawed three times in liquid nitrogen. The disrupted cells were mixed thoroughly with 0.1 mL of 5.0% (w/v) TCA containing 4.0 mM EDTA and centrifuged as above. The sediment was discarded, and 10 μL of supernatant was mixed with 300 μL of 0.1 M Tris-acetate buffer, pH 7.75. Aliquots were used for the determination of ATP, ADP and AMP. Standards containing known amounts of ATP were used in every experiment.

Lipid peroxidation assay. Lipid peroxidation in whole cells was determined by measuring the *in vitro* formation of TBA-reactants, essentially as described by Stacey and Kappus [25]. Hepatocytes were incubated in Krebs–Henseleit buffer (pH 7.4) supplemented with 5.0 mM glucose and additions as indicated under Results. Incubations were performed in the New Brunswick Shaker as above, at 37° and 90 cycles/min, under 95% O_2 + 5% CO_2 atmosphere. Duplicate samples (1.0 mL) of the incubation mixture were taken at the times indicated under Results, and 0.67 mL of 20% (w/v) TCA was added. After centrifugation, 0.33 mL of 0.67% (w/v) TBA in 0.05 N NaOH was added to 1.0 mL of supernatant. Samples were heated at 100° for 10 min, and

Table 1. Effect of NI on ATP, ADP and AMP levels in hepatocytes

NI (μM)	Adenine nucleotide	Time of incubation (hr) 0	1	2	3	4
0	ATP	20.50 ± 0.50	19.50 ± 0.77	18.63 ± 0.48	12.46 ± 0.43*	8.78 ± 0.41†
	ADP	3.99 ± 0.26	3.78 ± 0.19	3.84 ± 0.18	3.29 ± 0.16	3.45 ± 0.26
	AMP	0.98 ± 0.05	0.93 ± 0.06	0.90 ± 0.08	1.83 ± 0.06*	3.16 ± 0.42†
25	ATP	21.36 ± 0.79	19.39 ± 1.07	17.33 ± 0.56*	10.03 ± 0.28‡	4.35 ± 0.31†
	ADP	3.92 ± 0.11	3.83 ± 0.07	3.86 ± 0.09	3.46 ± 0.27	3.22 ± 0.15
	AMP	1.01 ± 0.03	1.12 ± 0.02	1.06 ± 0.02§	3.08 ± 0.11†	4.31 ± 0.44*
50	ATP	20.01 ± 0.30	15.88 ± 0.24‡	15.40 ± 0.37†	6.56 ± 0.54*	3.63 ± 0.36‡
	ADP	3.78 ± 0.03	3.75 ± 0.01	4.01 ± 0.05	3.54 ± 0.29	3.84 ± 0.06
	AMP	1.00 ± 0.07	1.39 ± 0.06§	1.85 ± 0.12*	3.61 ± 0.15*	4.80 ± 0.24†
100	ATP	19.38 ± 0.86	13.30 ± 0.91†	13.00 ± 0.58†	4.73 ± 0.39†	0†
	ADP	3.76 ± 0.24	3.51 ± 0.18	3.90 ± 0.24	3.44 ± 0.28	3.21 ± 0.20
	AMP	0.93 ± 0.06	1.60 ± 0.05*	2.08 ± 0.08‡	4.02 ± 0.13†	5.11 ± 0.12†

Experimental conditions were as described under Materials and Methods. Values are expressed in nmol nucleotide per 10^6 hepatocytes and are the means ± SEM of at least eight duplicate independent measurements.

*–§ Significantly different at the following levels: *$P < 0.025$, †$P < 0.005$, ‡$P < 0.01$, and §$P < 0.05$. P values for 0 μM NI were calculated with respect to the 0 time value.

absorbance was measured at 532 nm. The amount of TBA reactive products was calculated using $\varepsilon = 1.56 \times 10^5 M^{-1} \cdot cm^{-1}$. NADPH- and *t*-BuOOH-dependent lipid peroxidation in microsomes was measured as described [19, 20]. For the assay of NADPH-dependent lipid peroxidation, the incubation mixture contained liver microsomes (1.5 mg protein/mL), the NADPH-generating system (0.55 mM $NADP^+$, 5.5 mM G6P, 1.2 U/mL G6PD, 5.5 mM $MgCl_2$), 1.7 mM ADP, 0.1 mM $FeCl_3$, 130 mM KCl and 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4. For the *t*-BuOOH-induced peroxidation, the incubation mixture contained 2.6 mM *t*-BuOOH, microsomes (1.5 mg protein/mL), 0.11 mM EDTA, 0.1 mM $FeSO_4$, 130 mM KCl and 23 mM KH_2PO_4-Na_2HPO_4 buffer, pH 7.4. Mitochondria were incubated in 150 mM KCl, 50 mM Tris–HCl buffer, pH 7.4, supplemented with 2.5 mM NADH or 0.11/0.10 mM EDTA/Fe(III) plus 2.5 mM NADH, as indicated under Results.

Measurements of respiration rates. Respiration rates were measured polarographically, with a model 5/6 Oxygraph (Gilson Medical Electronics, Middleton, WI, U.S.A.), fitted with a Clark oxygen electrode, at 30°. For mitochondrial respiration, the standard reaction mixture (1.8 mL) contained mitochondria (1.0 to 2.0 mg protein/mL), 0.24 M sucrose, 34 mM KCl, 5.0 mM $MgCl_2$, 0.9 mM EDTA, 9.0 mM Tris–HCl, and 6.0 mM Na_2HPO_4-KH_2PO_4 buffer, pH 7.4. Unless stated otherwise, substrate concentrations were as follows (mM): L-malate, 5.0 and L-glutamate, 5.0 (plus malonate, 2.5); succinate, 10 (plus 3 μM rotenone), and NADH, 2.5. The rate of mitochondrial respiration was measured in the metabolic State "3," after adding 0.5 mM ADP. The respiratory control index was calculated as the ratio of State "3"/State "4" respiration.

Measurement of swelling. The degree of swelling was measured at 30° by following the absorbance change of the mitochondrial suspension [26] at 540 nm, with a Perkin Elmer 550S UV/VIS spectrophotometer. The rate of swelling was calculated from the slope of the corresponding tracings and is expressed as ΔA per minute per milligram of mitochondrial protein. Maximal swelling was calculated as the difference between absorbance before swelling and the absorbance value reached after swelling stabilization.

Dual wavelength spectrophotometry. The redox levels of cytochromes ($c + c_1$) and *b* were measured with a DW 2a UV/VIS spectrophotometer at 30° [27]. Other experimental conditions are described in the legend of Fig. 1. When measuring cytochromes $c + c_1$, absorbance was measured at 552 nm, a value intermediate between the maxima of *c* (550 nm) and c_1 (553 nm) [27]. Cytochrome *b* was measured at 430–410 nm, the peak and the trough of the Soret band [27]. NI was added after the reducing substrate (succinate), since at the selected wavelength NI absorbance did not interfere with the measurement of the redox level of the cytochromes. With cytochrome *b*, it was necessary to add NI before succinate in order to compensate for NI absorbance before measuring the cytochrome absorbance variation.

Assay of ATPase activity. ATPase activity was measured at 30° in an incubation mixture (final volume, 1.0 mL) containing 0.15 M Tris–HCl (pH 7.6), 3.0 mM ATP, 4.0 mM $MgCl_2$ and 0.25 mM EGTA. The reaction was started by adding the mitochondria. After 20 min of incubation, 0.1 mL of 50% (w/v) TCA was added. Orthophosphate concentration was determined by the method of Fiske and Subbarow [28].

Protein concentration. This was measured by the method of Gornall *et al.* [29].

Expression of results. When the values presented were the average of duplicate measurements, the experimental values deviated from the mean by less than 5%. When more than two samples were

Table 2. Effects of NI and nifurtimox (NX) on the adenylate system parameters

Nitro compound (μM)	Time of incubation (hr)				
	0	1	2	3	4
Parameter: Adenylate energy charge (ATP + 0.5 ADP)/(ATP + ADP + AMP)					
NI (0)	0.88 ± 0.03	0.88 ± 0.04	0.88 ± 0.03	0.80 ± 0.03	0.67 ± 0.04*
NI (25)	0.89 ± 0.04	0.88 ± 0.06	0.87 ± 0.03	0.71 ± 0.02†	0.50 ± 0.04‡
NI (50)	0.88 ± 0.02	0.84 ± 0.02	0.82 ± 0.02	0.61 ± 0.05‡	0.45 ± 0.03§
NI (100)	0.88 ± 0.05	0.82 ± 0.08	0.75 ± 0.04†	0.53 ± 0.04‡	0.19 ± 0.01§
NX (0)	0.89 ± 0.06	0.89 ± 0.04	0.88 ± 0.03	0.84 ± 0.03	0.83 ± 0.03
NX (25)	0.91 ± 0.04	0.83 ± 0.02	0.83 ± 0.03	0.83 ± 0.03	0.81 ± 0.03
NX (50)	0.89 ± 0.04	0.83 ± 0.03	0.83 ± 0.05	0.81 ± 0.04	0.80 ± 0.04
Parameter: ATP/ADP					
NI (0)	5.14 ± 0.36	5.16 ± 0.33	4.85 ± 0.26	3.78 ± 0.22‡	2.54 ± 0.22§
NI (25)	5.45 ± 0.25	5.06 ± 0.38	4.49 ± 0.18	2.90 ± 0.24§	1.35 ± 0.11‡
NI (50)	5.30 ± 0.29	4.23 ± 0.26	3.84 ± 0.10‡	1.85 ± 0.21§	0.94 ± 0.42§
NI (100)	5.15 ± 0.40	3.79 ± 0.32‡	3.53 ± 0.33‡	1.38 ± 0.15§	0§
Parameter: ATP + ADP + AMP					
NI (0)	24.9 ± 0.6	24.2 ± 0.8	23.3 ± 0.5	17.8 ± 0.5†	15.3 ± 0.6§
NI (25)	26.3 ± 0.8	24.3 ± 1.1	22.2 ± 0.6*	16.6 ± 0.4‡	11.9 ± 0.6§
NI (50)	24.8 ± 0.3	21.0 ± 0.4†	21.3 ± 0.4†	13.7 ± 0.9§	12.3 ± 0.4§
NI (100)	24.9 ± 1.1	18.4 ± 0.9‡	18.9 ± 0.6‡	12.2 ± 0.5§	8.7 ± 0.2§

Values were calculated from the mean ± SEM values given in Table 1.
* $P < 0.05$.
† $P < 0.025$.
‡ $P < 0.01$.
§ $P < 0.005$.

Table 3. Effect of NI on LDH leakage from rat hepatocytes

Time of incubation (hr)	LDH (mU/10^6 cells)		
	0 μM NI	50 μM NI	100 μM NI
0	0.91 ± 0.09	0.85 ± 0.09	1.16 ± 0.12
1	1.01 ± 0.10	0.96 ± 0.06	1.03 ± 0.01
2	1.13 ± 0.16	1.15 ± 0.10	1.46 ± 0.14
3	1.69 ± 0.09	1.58 ± 0.03	1.76 ± 0.47
4	1.91 ± 0.25	2.62 ± 0.24*	3.49 ± 0.09*

Experimental conditions were as described under Materials and Methods. Values are means ± SEM; N ≥ 3.
* $P < 0.005$.

measured, values are presented as means ± SEM. Statistical analysis was performed using Student's *t*-test for paired samples. Unless stated otherwise, the effect of NI was calculated by taking as the control value the nucleotide level (or activity) in the DMFA-containing sample, at the same incubation time.

RESULTS

Effect of NI on hepatocyte adenylate charge. Incubation of hepatocytes in the presence of NI produced depletion of intracellular ATP in a time- and concentration-dependent manner (Table 1). Under the various experimental conditions, the kinetics of ATP depletion were biphasic. After the first 2 hr of incubation (the "first period"), the drop in the ATP level was 7 ($P < 0.025$), 17 and 30% ($P < 0.005$) with 25, 50 and 100 μM NI, respectively, as compared with the DMFA-containing sample, at the same incubation time. During the last 2 hr of incubation (the "second period"), the rate of ATP depletion increased in all samples. After 4 hr of incubation with 25, 50 or 100 μM NI, the drop of the ATP level was 50 ($P < 0.005$), 59 ($P < 0.01$) and 100% ($P < 0.005$), as compared with the DMFA-containing sample. A variation in ATP should imply a contrary variation in ADP and/or AMP and, accordingly, the three nucleotides were measured. The results in Table 1 may be described as follows: (a) in control hepatocytes, nucleotide levels did not vary significantly during the first incubation period, but the ATP level decreased and the AMP level increased over the second period; (b) 25, 50 and 100 μM NI decreased the ATP level and increased the AMP level, during both the first and the second periods; (c) under the same experimental conditions, the ADP level did not vary to a significant degree; (d) in control hepatocytes, DMFA did not affect ATP, ADP and AMP levels significantly during the first incubation period, but decreased the ATP level 28% and increased the AMP level 11% during the second period, as compared with a system lacking DMFA (results not shown).

ATP, ADP and AMP constitute the cellular adenylate system, whose energy charge plays an important regulatory role in enzyme activity and cell metabolism [7]. Values calculated from the data in Table 1 are presented in Table 2. It is to be seen that (a) the adenylate energy charge of control hepatocytes did not vary during the first period of incubation but decreased ($P < 0.05$) during the

Table 4. Effect of NI on hepatocyte respiration: influence of antimycin

NI (μM)	Rate of respiration (ng-atoms 0/min per 10^6 cells) Without antimycin (A)	Plus antimycin (B)	A − B
None	25.9 ± 2.5	14.2 ± 0.5	11.7
25	24.1 ± 3.2	10.6 ± 2.0	13.5
50	22.6 ± 3.0	11.1 ± 2.1	11.5
100	21.4 ± 2.8	12.1 ± 2.0	9.3
200	16.8 ± 2.1*	12.4 ± 1.5	4.4

Hepatocyte respiration was measured with the Gilson Oxygraph model 5/6. The reaction mixture contained 6×10^5 cells/mL suspended in the Krebs–Henseleit buffer mixture, supplemented with 12.5 mM HEPES, pH 7.4, and saturated with oxygen. Antimycin (25 μM) and NI were added as indicated above. Values are means ± SEM of three independent measurements, each in duplicate.
* $P < 0.05$.

Table 5. Effect of NI on substrate oxidation by liver mitochondria

NI (μM)	Rate of oxidation (ng-atoms 0/min per mg protein) State "3" (A)	State "4" or "3u" (B)	A/B
	Substrate: 5.0 mM L-malate + 5.0 mM L-glutamate		
None	81.9 ± 2.7	19.3 ± 2.5	4.47 ± 0.57
25	71.3 ± 6.6	20.9 ± 2.8	3.68 ± 0.64
50	54.1 ± 4.5*	22.4 ± 3.4*	2.65 ± 0.47†
100	39.9 ± 0.5‡	26.0 ± 5.4‡	1.71 ± 0.28*
200	30.0 ± 4.3‡	31.1 ± 6.3‡	1.00 ± 0.08‡
	Substrate: 10 mM succinate		
None	156 ± 5.9	39.3 ± 2.7	4.05 ± 0.34
25	169 ± 3.8	47.0 ± 3.8	3.68 ± 0.32
50	166 ± 6.8	54.8 ± 4.2§	3.10 ± 0.30
100	157 ± 9.9	67.8 ± 6.5‡	2.39 ± 0.25‡
200	141 ± 7.8	87.6 ± 6.4‡	1.67 ± 0.20‡
Control	160 ± 15.6	41.1 ± 2.6	4.31 ± 0.38

Experimental conditions were as described under Materials and Methods. Values are means ± SEM of four duplicate independent measurements. Control, sample without DMFA.
* $P < 0.005$.
† $P < 0.05$.
‡ $P < 0.01$.
§ $P < 0.025$.

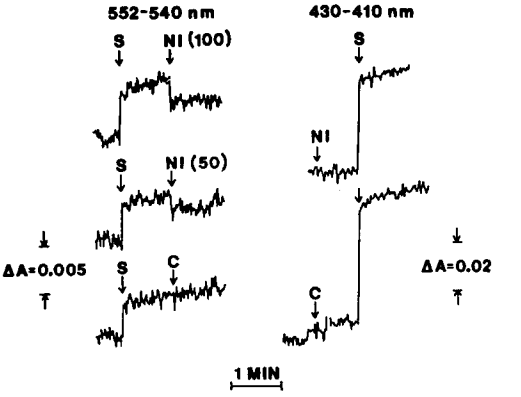

Fig. 1. Effect of NI on redox levels of cytochromes ($c + c_1$) and b. The reaction medium was as in Table 5 and contained 3.0 μM rotenone and 0.5 mg mitochondrial protein per mL. Succinate (10 mM) (S) and NI (100 μM, unless stated otherwise) were added as indicated on the figure. C (control) indicates 40 μL of DMFA, the same volume of the NI solution. The wavelength pairs used are indicated on the figure. Other experimental conditions were as described under Materials and Methods. The upward and downward shifts of tracings indicate cytochrome reduction and oxidation, respectively. When measuring cytochrome b reduction, variation of absorbance was compensated for after adding NI.

second period; (b) with the NI-treated hepatocytes, the adenylate energy charge decreased significantly, particularly in the second incubation period with 25, 50 and 100 μM NI; (c) under similar experimental conditions, the nitrofuran derivative nifurtimox did not decrease the adenylate energy charge to a significant degree; (d) the ATP/ADP ratio, which reflects the phosphorylating potential, varied in close correlation with the energy charge; (e) total adenylate content of NI-treated hepatocytes decreased significantly with 25, 50 and 100 μM NI, during both incubation periods.

It is known that hepatocyte viability depends on intracellular ATP, among several other variables [6]. Accordingly, it seemed of interest to establish the effect of NI on LDH leakage [12], a parameter of hepatocyte viability. Leakage of LDH from control and NI-treated hepatocytes was measured, and the corresponding results are shown in Table 3. It can be seen that with the NI-treated hepatocytes, LDH leakage did not increase above the control level, except after 4 hr of incubation with 50 and 100 μM NI. DMFA did not affect LDH leakage as compared with a system lacking DMFA (experimental data not shown).

Effect of NI on electron transfer and oxidative phosphorylation in hepatocytes. Regulation of the ATP level in hepatocytes depends on mitochondrial oxidative phosphorylation [30, 31] and, therefore, NI action on mitochondrial electron transfer was examined. Table 4 shows the effect of NI on antimycin-sensitive and -insensitive hepatocyte respiration. The latter was not affected significantly by NI, but the former was inhibited 20 and 69% by 100 and 200 μM NI, respectively (A − B values; Table 4).

Table 5 shows the effect of NI on the respiration rate of coupled mitochondria, with MGM or succinate as substrate. It can be seen that (a) with MGM as substrate and mitochondria in metabolic State "3" (active or uncontrolled respiration [32]), NI produced significant inhibition: 34, 51 and 63%, at 50, 100 and 200 μM NI; (b) with the same substrate

Table 6. Effects of nitro compounds on mitochondrial NADH-oxidase activity

Nitro compound (μM)	Antimycin (μg/mg protein)	Oxygen uptake (ng-atom/min per mg protein)
None	0	47.1
NI (50)	0	41.4 (12)
NI (100)	0	29.1 (38)
NI (200)	0	23.0 (51)
Nitrofurantoin (220)	0	46.8 (0.4)
Nitrofurazone (200)	0	45.0 (4.4)
NF-pyrazole (200)	0	66.2
NF-triazole (200)	0	63.8
None	1	7.3
NI (100)	1	7.3 (0)
NF-pyrazole (100)	1	23.1
NF-triazole (100)	1	37.1

Oxygen uptake was measured polarographically. The experimental conditions were as described under Materials and Methods. The reaction mixture contained disrupted mitochondria (20 mg/mL protein), 2.5 mM NADH and nitro compound, as indicated above. Values are averages of at least duplicate measurements. The percent inhibition of oxygen uptake is given in parentheses.

Table 7. Effects of nitro compounds on ascorbate oxidation

Nitro compound (μM)	O_2 uptake μMO_2 min . mmol compound
None	0
Nifurtimox (300)	32 ± 2
Nitrofurantoin (200)	29 ± 2
Nitrofurazone (200)	33 ± 2
NI (220)	3 ± 2

The reaction mixture contained 5.0 mM sodium ascorbate, 1.0 mM DETAPAC, 0.1 M Tris–HCl buffer, pH 7.5, and nitro compound as indicated above. Oxygen uptake was measured polarographically [17]. Experimental conditions were as described in Ref. 17. Values are means ± SEM of at least three duplicate independent measurements.

mixture and mitochondria in metabolic State "4," NI activated respiration to a significant degree (1.5-fold, with 200 μM NI), thus inducing the metabolic State "3u" ("3u" [32]); (c) as a result of the opposite variations of respiration in States "3" and "3u," the State "3"/State "4" (or "3u") index decreased significantly; (d) with succinate as substrate and mitochondria in State "3," NI did not affect the respiration rate but with mitochondria in State "4," NI activated succinate oxidation significantly (2.2-fold).

In close agreement with the effect of NI on mitochondrial respiration in metabolic State "4" (Table 5), addition of NI to mitochondria oxidizing succinate in the absence of ADP shifted the redox levels of reduced cytochromes $c + c_1$ and b towards the oxidized state. A typical experiment is described in Fig. 1. The effect of NI on cytochromes $c + c_1$ was concentration dependent, in close agreement with results in Table 5.

NI was also assayed on electron transfer in disrupted mitochondrial membranes. The results presented in Table 6 show that in the absence of antimycin, NI inhibited NADH oxidation in a concentration-dependent manner, unlike the nitrofuran derivatives nitrofurazone and nitrofurantoin, which inhibited only slightly or not at all, respectively. Moreover, the nitrofuran derivatives NF-pyrazole and NF-triazole, which in the presence of adequate electron donors and oxygen catalyze oxygen consumption and oxygen radical production [17, 18], increased oxygen uptake catalyzed by mitochondrial membranes, irrespective of the presence of antimycin. The results shown in Table 6 prove that the assayed mitochondrial membranes could catalyze nitro group redox-cycling, but this catalysis did not occur with NI. These observations were confirmed using ascorbate as electron donor (Table 7).

Effect of NI on latent ATPase activity. F_1F_0-ATP synthase is an essential component of the mitochondrial energy-transducing system. Taking into account the results described in Table 5, it seemed of interest to examine NI action on the enzyme latent ATPase activity. The results in Table 8 show that 100 and 200 μM NI stimulated ATP hydrolysis, in contrast to the results obtained with the disrupted mitochondrial membranes, whose ATPase activity was not modified to any significant degree by NI.

Effect of NI on mitochondrial swelling. In the presence of permeant anions, Ca^{2+} is accumulated rapidly by mitochondria in an energy-dependent process that induces swelling and H^+ ejection [33, 34]. Figure 2 shows that (a) with MGM as substrate (tracings A and B), swelling was inhibited 84% by NI; (b) the endogenously driven swelling was stimulated by NI (tracings C and D); and (c) the succinate-driven swelling was inhibited 25% by NI (tracings E and F). In every case, EGTA blocked mitochondrial swelling, thus proving the role of

Table 8. Effect of NI on ATP hydrolysis by coupled and uncoupled mitochondria

NI (μM)	ATPase activity (mU/mg protein)	
	Coupled mitochondria	Disrupted mitochondria
None	32 ± 9	175 ± 45
25	33 ± 9	176 ± 40
50	39 ± 8	180 ± 50
100	49 ± 5*	174 ± 50
200	67 ± 7†	175 ± 60

F_1F_0-ATP synthase ATPase activity was measured by the rate of ATP hydrolysis. The reaction mixture contained 0.32 to 0.68 mg/mL mitochondrial protein. Other conditions were as described under Materials and Methods. Values are means ± SEM of four duplicate independent measurements.

* $P < 0.01$.

† $P < 0.001$.

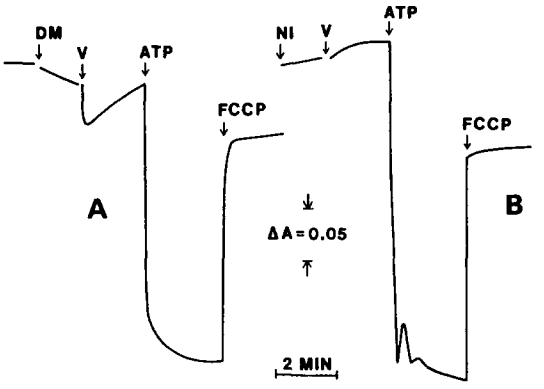

Fig. 3. Effect of NI on mitochondrial swelling after valinomycin addition. The reaction medium was as described in the legend of Fig. 2, except that rotenone was omitted. Mitochondria: 0.75 mg protein/mL. NI (200 μM), valinomycin (V; 80 nM), ATP (2.0 mM), DMFA (DM; 40 μL) and FCCP (2 μM) were added as indicated on the figure. Other experimental conditions were as described under Materials and Methods. (A) control (DMFA) experiment; (B) NI experiment.

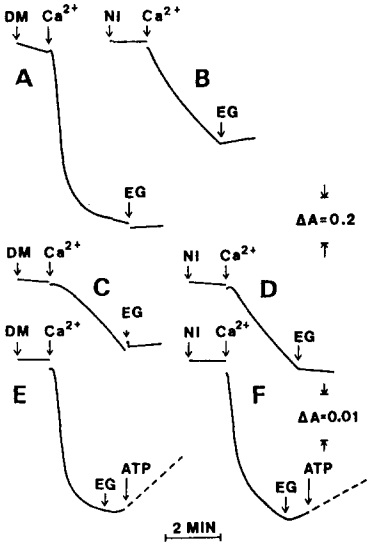

Fig. 2. Effect of NI on mitochondrial swelling after Ca^{2+} addition. Reaction medium: 0.1 M sucrose, 50 mM KCl, 20 mM 4-morpholinepropanesulfonic acid (MOPS), 0.2 mM KH_2PO_4, pH 7.2, 0.8 μM rotenone (only in experiments E and F) and 0.4 mg mitochondrial protein per mL. Substrate additions: A and B, 1.7 mM L-malate, 1.7 mM L-glutamate, 0.8 mM malonate; C and D, none; E and F, 5.0 mM succinate. NI (200 μM) was added as indicated on the figure, and control samples received the corresponding volume of DMFA (DM). $CaCl_2$ (Ca^{2+}; 120 μM), EGTA (EG; 0.1 mM) and MgATP (ATP; 2.5 mM) were added as indicated on the figure. The tracings after MgATP addition are read against the lower ΔA scale.

Ca^{2+} in the mitochondrial volume variation. The contraction of mitochondrial volume by EGTA and ATP (Fig. 2, tracings E and F) was inhibited 27% by NI. Table 9 summarizes the effect of increasing concentration of NI on Ca^{2+} uptake. It can be seen that with MGM as substrate, NI decreased the rate of swelling at the same concentrations that inhibited MGM oxidation (Table 5), whereas with succinate, NI only inhibited Ca^{2+} uptake at the 200 μM concentration.

Addition of valinomycin to mitochondria in the presence of K^+ and phosphate greatly accelerates the penetration of K^+, which can be driven by energy derived from substrate oxidation or ATP hydrolysis [35]. Figure 3 shows the results of a typical experiment, in which NI was assayed on the valinomycin-induced swelling. It can be seen that 200 μM NI completely inhibited swelling supported by the oxidation of endogenous substrates. On the other hand, NI inhibited the ATP-dependent swelling to a much lesser degree but no quantitative expression of this inhibition could be obtained since the rate of swelling induced by ATP was too fast to be measured. The effect of NI on the valinomycin-dependent swelling recalls previous results with steroids [26].

Effect of NI on lipid peroxidation. Lipid peroxidation processes play an important role in liver damage by many toxic agents [8–10]. Accordingly, the effect of NI on MDA production by hepatocytes was investigated. The results presented in Fig. 4 indicate that (a) in control hepatocytes, MDA production increased linearly as a function of incubation time; (b) NI inhibited MDA production, and this inhibition occurred at the same NI concentrations that affected the adenylate energy charge (50 and 100 μM NI). Since lipid peroxidation occurred in both microsomes and mitochondria, the effect of NI on MDA production by these organelles was also examined. The results in Table 10 show that NI inhibited microsomal lipid peroxidation, in close agreement with the effect on hepatocytes. The NADPH-initiated microsomal lipid peroxidation was strongly inhibited by NI but the *t*-BuOOH-dependent

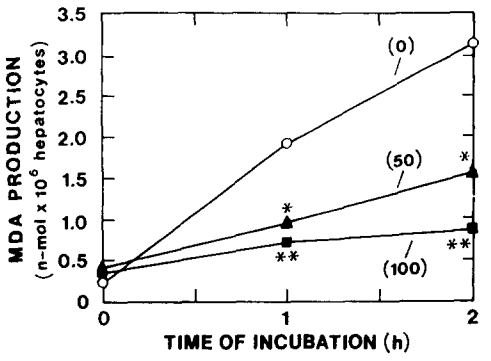

Fig. 4. Effect of NI on MDA production in hepatocytes. NI concentration (μM) was as indicated by the numbers in parentheses. Other experimental conditions were as described under Materials and Methods. Values are the means of at least three duplicate independent measurements, and were compared with the DMFA-containing sample at the same time of incubation. Key: (*) $P < 0.01$; and (**) $P < 0.001$.

peroxidation was less affected. Under similar experimental conditions, NADH-initiated mitochondrial lipid peroxidation was inhibited 27 and 34% by 50 and 100 μM NI, respectively, whereas the Fe-EDTA-stimulated lipid peroxidation was inhibited to a non-significant degree.

DISCUSSION

The observations described in the present study indicate that in addition to its mutagenic action [1–4], NI can produce metabolic alterations in whole liver cells, especially in the mitochondrial system, as illustrated by the modification of the adenylate energy charge (Tables 1 and 2).

The adenylate energy charge plays an essential role for intracellular homeostasis [30, 31], and its variations can be visualized by using Atkinson's relationship [7] or the ATP/ADP ratio (Table 2). It is generally agreed that for reactions that participate in sequences utilizing ATP, plots of enzyme activity against energy charge have positive slopes that increase with charge [36]. On the other hand, for reactions that participate in sequences regenerating ATP, plots of enzyme activity against energy charge have negative slopes that increase in absolute value

Table 9. Effect of NI on mitochondrial swelling after Ca^{2+} addition

Substrate	NI (μM)	Swelling parameters: Initial rate $\left(\frac{\Delta A/\text{min}}{\text{mg protein}}\right)$	Swelling parameters: Maximal swelling $\left(\frac{\Delta A}{\text{mg protein}}\right)$
L-Malate-L-glutamate (MGM)	None	2.13	1.07
	50	1.74	1.11
	100	0.88 (59)	1.04
	200	0.34 (84)	0.65 (40)
Succinate	None	0.52	0.29
	100	0.49 (5)	0.29
	200	0.39 (25)	0.27

Experimental conditions were as described in the legend of Fig. 2. Values are the means of at least two measurements. The percent inhibition of mitochondrial swelling is given in parentheses.

Table 10. Effect of NI on lipid peroxidation in microsomes and mitochondria

NI (μM)	MDA production (nmol/mg protein): Microsomes: NADPH-generating system	Microsomes: *t*-BuOOH system	Mitochondria: NADH	Mitochondria: NADH + EDTA-Fe(III)
0	31.1	9.83	3.03	18.8
50	1.75 (94)	7.32 (26)	2.20 (27)	18.3 (2.6)
100	1.56 (95)	5.63 (43)	2.0 (34)	17.5 (6.9)

Experimental conditions were as described under Materials and Methods. Incubation time: 60 min (with NADPH-generating system or mitochondria) or 15 min (with *t*-BuOOH system). Data represent the averages of duplicate measurements. The percent inhibition of MDA production is given in parentheses.

with charge [37]. With *Escherichia coli* enzymes, variation of charge values in the 0.7 to 1.0 range produced maximum variation of enzyme activity [36, 37]. The energy charge variations produced by NI (Table 2) fit in well with (a) those affecting regulated enzymes [36, 37] and (b) those produced by specific inhibitors of glycolysis or oxidative phosphorylation [6, 30, 31]. Interestingly enough, the antichagasic nitrofuran nifurtimox was a much less effective modifier of the adenylate energy charge than NI was (Table 2), despite its capability of redox cycling and oxyradical production in liver preparations [38].

ATP depletion, lipid peroxidation and loss of viability are closely related phenomena in hepatocytes [6, 39]. As regards ATP, it is known that depletion of ATP may cause deleterious effects on many cellular activities, such as the maintenance of ionic gradients, the polymerization of actin, and the maintenance of the cytoskeleton [39]. Since NI inhibited lipid peroxidation (Fig. 4 and Table 10), no significant role should be attributed to lipid peroxidation products as intermediates of NI action. As regards the release of LDH from NI-treated hepatocytes, which indicates loss of cell viability, it is generally agreed that it follows the drop of cytosolic ATP [6, 39]. NI increased LDH release, especially with 50 or 100 μM NI after 4 hr of incubation (Table 3). The relatively low correlation between decrease of energy charge and cell viability during the first 2 hr of incubation (Tables 2 and 3) may be explained by assuming that NI affected intramitochondrial ATP production. This ATP would be less effective than its cytosolic counterpart in controlling plasma membrane stability [6].

The different results obtained with NI and nifurtimox demonstrate that oxygen radical production by nifurtimox redox-cycling should not affect oxidative phosphorylation in hepatocytes. In this connection, it must be noted that nifurtimox was used at a concentration greater than that existing in the blood of patients receiving nifurtimox. The relative insensitivity of the evaluated parameters to this nitrofuran derivative may be explained by the existence in hepatocytes of protective mechanisms against oxyradicals [40, 41].

Inhibition of mitochondrial electron transfer and oxidative phosphorylation may contribute to the toxic action of NI on hepatocytes. This inhibition has not been described before, and therefore it seems pertinent to summarize its main features. Thus, (a) NI inhibited electron transfer at Site I, as shown by the effect of substrates in Table 5; (b) inhibition of electron transfer also occurred with disrupted mitochondrial membranes (Table 6), which indicated a direct action on the electron transport mechanism, in all probability at the NADH-dehydrogenase flavoprotein; (c) at equivalent NI concentrations, the inhibition of electron transport in disrupted mitochondrial membranes was less than with intact mitochondria (Tables 5 and 6), which suggests a minor contribution of energy transfer inhibition to the action of NI on mitochondria; (d) NI half-maximal inhibitory concentration for intact mitochondria was about 100 μM (Table 5), which means that NI was a much weaker inhibitor than rotenone, piericidin and steroids [42]; (e) NI was not involved in redox-cycling reactions at Site I, at variance with nitrofuran compounds, in all probability because of different electronic molecular structures (Table 7); (f) inhibition of electron and energy transfer at Site I prevented energization of Ca^{2+} uptake by mitochondria and, consequently, swelling (Fig. 2 and Table 9); (g) like typical uncouplers of oxidative phosphorylation [32], NI released controlled substrate respiration (Table 5), shifted the redox level of reduced cytochromes (Fig. 1), released latent ATPase activity (Table 8) and prevented valinomycin-dependent uptake of K^+ (Fig. 3), like other inhibitors of electron transfer at Site I, e.g. androsterone [26].

Taken together, the summarized effects of NI support a complex action on the mitochondrial energy-conserving mechanisms, which involves inhibition of electron transfer and uncoupling of oxidative phosphorylation. NI effects on mitochondria account satisfactorily for NI action on hepatocytes, especially on the adenylate energy charge.

Finally, NI inhibition of Ca^{2+} deserves special comment. The maintenance of a low cytosolic free-Ca^{2+} concentration is a common feature of all eukaryotic cells [34]. For this purpose, several mechanisms ensure the buffering of Ca^{2+} in the cytoplasm and its accumulation within organelles. Mitochondrial Ca^{2+} transport plays an essential role in mitochondrial metabolism and, therefore, prevention of Ca^{2+} uptake by NI might contribute to its toxicity in hepatocytes.

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