Increasing concentrations (1-100 μM) of the redox cycling quinone, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), stimulated growth, triggered apoptosis, or caused necrosis of pancreatic RINm5F cells, depending on the dose and duration of the exposure. Following the exposure of RINm5F cells to 10 μM DMNQ, ornithine decarboxylase activity and polyamine biosynthesis increased. This was accompanied by enhanced cell proliferation. Conversely, exposure to 30 μM DMNQ for 3 h resulted in the inhibition of ornithine decarboxylase, intracellular polyamine depletion, and apoptotic cell killing. Pretreatment of the cultures with the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate, restored polyamine levels and prevented apoptosis. Exposure to the same DMNQ concentration for only 1 h, with subsequent re-incubation in growth medium, neither caused polyamine depletion nor resulted in apoptosis. Finally, exposure to an even higher DMNQ concentration (100 μM) for either 1 or 3 h caused rapid intracellular Ca2+ overload, ATP, NAD+, and glutathione depletion, and extensive DNA single strand breakage, which resulted in necrotic cell death.

Our results show that a disturbance of polyamine biosynthesis occurred prior to cell growth or apoptosis elicited by oxidative stress. In addition, we show that effects as opposite as cell proliferation and deletion, by either apoptosis or necrosis, can be induced, in the same system, by varying the exposure to a prooxidant.

In several pathophysiological conditions, excessive generation of free radicals can lead to various degrees of oxidative stress. Moderate oxidative stress may selectively alter sensitive physiological processes, including cell signaling and gene activation (1), as well as the balance between DNA damage and repair (2). Radicals can modulate the activity of kinases (3-5), promoting cell growth (6-8), and directly interact with protooncogenes (1). Thus, sustained exposure to moderate prooxidant levels may play an important role at several stages in carcinogenesis (9, 10). Conversely, when radical generation overwhelms cell antioxidant defense, lethal mechanisms are activated, and cell death ensues (11, 12). Excessive oxidative stress is known to cause cell necrosis (13), but it has been also implicated in the activation of apoptosis in several systems, including cytokine-dependant killing of human immunodeficiency virus-infected cells (14), neural cell killing following glutathione depletion (15), and macrophage apoptosis induced by the fungal metabolite, gliotoxin (16). More recently, it has also become apparent that genes that suppress apoptosis may encode for antioxidant proteins (17). Thus, it appears that oxidative stress, which alters both signal transduction and genomic processes, may cause either inappropriate growth stimulation or activate the cell death program in different experimental systems.

Increasing evidence suggests that growth stimulation, besides triggering the entering of cells into the cell cycle, can act as a surviving factor (18). Thus, cell cycle block can result in apoptosis (19), whereas mitotic stimuli can prevent apoptosis (20). Consequently, should mitosis and cell death be regulated by related processes, it is conceivable that one or more regulatory proteins are differentially expressed or activated in the normal cell cycle progression and in apoptosis. Ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, is induced after mitogenic stimulation of quiescent cells (21) and, in general, in cell proliferation (22). Induction of ODC and the other rate-limiting enzymes in polyamine biosynthesis, S-adenosyl-l-methionine decarboxylase (SAMDC) results in increased levels of intracellular putrescine, spermidine, and spermine. Increased intracellular levels of these polyamines in dividing cells may have multiple functions, including a possible antioxidant effect (23) and a modulatory effect on chromatin structure (24). We have previously reported that supplementation of the culture medium with polyamines prevents thymocyte apoptosis, whereas inhibition of polyamine biosynthesis can sensitize cells to apoptosis (25). Accordingly, it has been proposed that mitogenic stimulation may prevent the activation of the cell death program by increasing ODC expression and the intracellular polyamine content (26).

Since oxidative stress can either mimic mitogenic stimulation or cause cell death, we decided to study the effects of different concentrations of a redox cycling quinone, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), on cell survival and polyamine biosynthesis in an insulin-producing, pancreatic β cell line. We initially observed that the level of prooxidant and

*This work has been supported by grants from The Swedish Natural Science Research Council (NFR, Project 10173-300), The Swedish Medical Research Council (MFR, 03X-2471). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 46-8-728-7568; Fax: 46-8-314-2051.

1 The abbreviations used are: ODC, ornithine decarboxylase; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; Fura-2, 1-(2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl-2'-2'-amino-5'-methylphenoxy)-ethane-N,N',N''-tetracetic acid; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; Fura-2, 1-(2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl-2'-2'-amino-5'-methylphenoxy)-ethane-N,N',N''-tetracetic acid; Fura-2/AM, is the acetoxymethylester of Fura-2; HBS, Hepes-buffered saline; MeSO, dimethyl sulfoxide; pH, 8-hydroxydeoxyguanosine; SAMDC, S-adenosyl-l-methionine decarboxylase; TPA, 12-O-tetradecanoylphorbol-13-acetate; HPLC, high performance liquid chromatography.
duration of exposure to the quinone determined whether these cells were stimulated to grow, or to die by either apoptosis or necrosis. When the effects of DMNQ on polyamine biosynthesis were investigated, we found that intracellular polyamine depletion was closely associated with the onset of apoptosis. Preventing polyamine depletion by phorbol ester treatment also prevented apoptosis and restored cell proliferation.

EXPERIMENTAL PROCEDURES

Chemicals—DMNQ was a gift from Dr. Gerald Cohen, Medical Research Council, Toxicology Unit, Leicester, UK. The synthesis and properties of this redox cycling quinone are described in Gant et al. (27). Fura-2 AM and TPA were purchased from Sigma. [3H]Thymidine (NEC 568), [3H]Tritiated thymidine (CFA 4911), and 5-adenosyl-l-[carboxyl-14C]methionine (CFA 477) were obtained from Dupont-NEN and Amersham Corp., respectively. Standards of och/AG were a gift of Dr. Christopher Richter, Department of Biochemistry, ETH, Zurich. All other chemicals were commercial products of the highest available grade of purity.

Cell Cultures and Treatments—RINm5F cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% dialyzed fetal calf serum, 1% (v/v) l-glutamine, and 1% (v/v) penicillin-streptomycin (all media and media supplements were from Life Technologies, Inc.) (28). In all experiments, cells were seeded at a density of 20 × 10^3 cells/cm^2 and grown for 72 h to a density of approximately 0.5 × 10^5 cells/cm^2.

Cells were subsequently exposed in appropriate volumes of medium in cell culture vessels (Costar). DMNQ stocks were prepared by dilution with methanol. The final MeSO concentration during exposure was always <0.1%. Control cells were exposed to MeSO alone in all experiments.

Colony-Forming Efficiency—RINm5F cells were cultured in 96-well plates as described above and subsequently exposed to DMNQ. After exposure, the cells were washed once in growth medium and then reincubated for 3 h in fresh medium to allow for completion of DNA repair. After that, cells were washed, trypsinized, and subcultured at clonal density (250 cells/cm^2) in 60-mm dishes. Feeding was performed every 2 days. At each time point, the experiment was performed in triplicate, whereas control cells were stimulated to grow, or to die by either apoptosis or necrosis. When the effects of DMNQ on polyamine biosynthesis were investigated, we found that intracellular polyamine depletion was closely associated with the onset of apoptosis. Preventing polyamine depletion by phorbol ester treatment also prevented apoptosis and restored cell proliferation.

Alkaline Elution—RINm5F cells were cultured in six-well plates in the presence of [3H]thymidine (0.2-0.4 μCi/ml) for two generations. A chase of 24 h followed, when [3H]thymidine was substituted with cold thymidine. Cells were then exposed to DMNQ. DNA single-strand breakage was measured by the alkaline elution technique described by Kohn et al. (29). Results were calculated and expressed as the double logarithmic plot, normalized with respect to the elution of an internal standard (Hepes-5-adenylated L-2120 cells irradiated with 3 Gy). DNA damage was determined by the percentage of cells with DNA damage.

Alkaline Elution—RINm5F cells were cultured in six-well plates in the presence of [3H]thymidine (0.02-0.04 μCi/ml) for two generations. A chase of 24 h followed, when [3H]thymidine was substituted with cold thymidine. Cells were then exposed to DMNQ. DNA single-strand breakage was measured by the alkaline elution technique described by Kohn et al. (29). Results were calculated and expressed as the double logarithmic plot, normalized with respect to the elution of an internal standard (Hepes-5-adenylated L-2120 cells irradiated with 3 Gy). DNA damage was determined by the percentage of cells with DNA damage.

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Induced stimulation of ODC activity (Fig. 2A). Finally, exposure to 100 µM DMNQ caused an immediate and persistent inhibition of ODC activity (Fig. 2C). The activity of ODC did not significantly vary in control cells over the 6 h period. Pretreatment of RINm5F cells with the phorbol ester TPA stimulated ODC activity in untreated controls, but it did not prevent ODC inhibition after exposure to 30 µM DMNQ (data not shown). Fig. 3 shows that SAMDC activity decreased only slightly after exposure to 30 µM DMNQ. However, here, pretreatment with TPA prior to DMNQ exposure markedly increased the enzyme activity.

The changes observed in the activities of ODC and SAMDC were accompanied by alterations in intracellular polyamine content. As shown in Fig. 4A, spermidine and spermine levels increased with a maximum at 3 h following treatment with 10 µM DMNQ. Conversely, in cells exposed to 30 µM DMNQ, the intracellular content of spermine decreased progressively during the first 3 h to about 50% of the control value (Fig. 4B).

Fig. 1. RINm5F cell growth and survival following exposure to DMNQ. Panel A, colony forming efficiency. Cells were exposed to DMNQ for 1 h (○—○) or 3 h (▲—▲) as described under “Experimental Procedures.” Closed symbols indicate pretreatment with 10 nM TPA: 3 h before DMNQ addition (●); 1 h before DMNQ addition (■). Inset, 3H-thymidine incorporation in RINm5F cells exposed to 10 µM DMNQ for 1 h (○—○). Each point represents the mean ± S.D. of four to six separate experiments (*p < 0.05). Panel B, lactate dehydrogenase leakage: DMNQ 30 µM (□—□); 100 µM (△—△). Results represent means ± S.D. from eight separate experiments.

TPA, and then exposed to 30 µM DMNQ for 3 h. TPA obviously stimulated growth of untreated cells when used alone. However, the combination of TPA pretreatment with exposure of the cells for 1 h to 30 µM DMNQ stimulated cell growth to an extent higher than that elicited by the single TPA treatment. At 100 µM DMNQ caused cell killing after 1 h exposure, resulting in decreased colony forming efficiency (Fig. 1, A and B). In this case, pretreatment with TPA had no effect.

Intracellular ODC, SAMDC Activities, and Polyamine Levels—When RINm5F cells were exposed to 10 µM DMNQ (Fig. 2A), ODC activity increased. A moderate initial increase was observed within the first 30 min, whereas a second marked activation occurred between 1 and 3 h. The activity of ODC subsequently declined but remained at a level that was 2- to 3-fold higher than that observed before the addition of DMNQ. Pretreatment of cells with cycloheximide, to inhibit protein synthesis, abolished the increase in ODC activity observed after exposure to 10 µM DMNQ (Fig. 2A). Pretreatment with staurosporin, an inhibitor of protein kinase C-blocked DMNQ-induced stimulation of ODC activity (Fig. 2A). Similar findings were obtained for SAMDC (data not shown).

At 30 µM, DMNQ increased ODC activity during the first 30 min. However, ODC activity declined after 1 h, and by 2 h was reduced to about 50% of that found before exposure (Fig. 2B).

DMNQ Induces DNA Damage in RINm5F Cells—Exposure of RINm5F cells to 10 or 30 µM DMNQ resulted in base oxidation, as shown by the increased amounts of ohsdG found in the nuclear DNA (Table I). Nevertheless, the accumulation of DNA single strand breaks after exposure to 10 or 30 µM DMNQ for 1 h was relatively modest (≤1.5 single strand breaks × 10^{-10} daltons). Extensive single strand breakage was instead found after 1 h exposure to 100 µM DMNQ (Fig. 6A). Extending to 3 h the exposure to either 10 or 30 µM DMNQ caused only a moderate increase in single strand break formation (1.7 single strand breaks × 10^{-10} daltons with 30 µM DMNQ). In contrast, agarose gel electrophoresis of DNA extracted from cells exposed to 30 µM DMNQ revealed a DNA fragmentation pattern indicative of internucleosomal cleavage (Fig. 6B), such as that caused by endonucleases during apoptosis (40). DNA fragmentation was evident at 3 and 6 h, as cells progressively lost adherence to the dishes, but did not release lactate dehydrogenase (see Fig. 1B). The events leading to apoptosis occurred between 1 and 3 h, since cells exposed to DMNQ for 1 h, washed, and then reincubated in fresh medium neither detached nor presented nuclear pyknosis and DNA fragmentation. Cells exposed to 10 µM DMNQ had no DNA fragmentation, whereas the DNA extracted from cells treated with 100 µM

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At 30 µM, DMNQ increased ODC activity during the first 30 min. However, ODC activity declined after 1 h, and by 2 h was reduced to about 50% of that found before exposure (Fig. 2B). Finally, exposure to 100 µM DMNQ caused an immediate and persistent inhibition of ODC activity (Fig. 2C). The activity of ODC did not significantly vary in control cells over the 6 h period. Pretreatment of RINm5F cells with the phorbol ester TPA stimulated ODC activity in untreated controls, but it did not prevent ODC inhibition after exposure to 30 µM DMNQ (data not shown). Fig. 3 shows that SAMDC activity decreased only slightly after exposure to 30 µM DMNQ. However, here, pretreatment with TPA prior to DMNQ exposure markedly increased the enzyme activity.

The changes observed in the activities of ODC and SAMDC were accompanied by alterations in intracellular polyamine content. As shown in Fig. 4A, spermidine and spermine levels increased with a maximum at 3 h following treatment with 10 µM DMNQ. Conversely, in cells exposed to 30 µM DMNQ, the intracellular content of spermine decreased progressively during the first 3 h to about 50% of the control value (Fig. 4B). Finally, putrescine depletion occurred rapidly, and cells contained only 40% of this polyamine after 1 h (Fig. 4C). Pretreatment of the cells with TPA for 1 h prior to the addition of 30 µM DMNQ increased the initial spermine and putrescine levels (Fig. 4, B and C). Moreover, TPA pretreatment prevented spermine depletion and delayed the loss of putrescine caused by 30 µM DMNQ (Fig. 4, B and C). The loss of putrescine after 3 h of incubation with DMNQ was not prevented by TPA treatment. Since many cells progressively detached and shrank with a typical apoptotic appearance (see below), during the first 6 h of incubation, we decided to measure the polyamine content in these cells and in the population that had remained attached to the dishes. We harvested detached cells between 3 and 6 h after treatment with 30 µM DMNQ. Virtually all these cells had the typical apoptotic morphology: a condensed cytoplasm and a highly fluorescent pyknotic nucleus (Fig. 5A). A few preapoptotic cells (i.e. with patched and partially condensed chromatin) could still be seen among the adherent cell population (Fig. 5B), whereas control cells had none of the above. In the apoptotic population, both putrescine and spermine levels were reduced to about 20% of those measured in the adherent population. In the apoptotic cells the putrescine and spermine contents were 18 ± 4 pmol/10^6 cells and 176 ± 20 pmol/10^6 cells, respectively, whereas the adherent population had 108 ± 28 pmol putrescine/10^6 cells and 812 ± 78 pmol spermine/10^6 cells.

DMNQ Induces DNA Damage in RINm5F Cells—Exposure of RINm5F cells to 10 or 30 µM DMNQ resulted in base oxidation, as shown by the increased amounts of ohsdG found in the nuclear DNA (Table I). Nevertheless, the accumulation of DNA single strand breaks after exposure to 10 or 30 µM DMNQ for 1 h was relatively modest (≤1.5 single strand breaks × 10^{-10} daltons). Extensive single strand breakage was instead found after 1 h exposure to 100 µM DMNQ (Fig. 6A). Extending to 3 h the exposure to either 10 or 30 µM DMNQ caused only a moderate increase in single strand break formation (1.7 single strand breaks × 10^{-10} daltons with 30 µM DMNQ). In contrast, agarose gel electrophoresis of DNA extracted from cells exposed to 30 µM DMNQ revealed a DNA fragmentation pattern indicative of internucleosomal cleavage (Fig. 6B), such as that caused by endonucleases during apoptosis (40). DNA fragmentation was evident at 3 and 6 h, as cells progressively lost adherence to the dishes, but did not release lactate dehydrogenase (see Fig. 1B). The events leading to apoptosis occurred between 1 and 3 h, since cells exposed to DMNQ for 1 h, washed, and then reincubated in fresh medium neither detached nor presented nuclear pyknosis and DNA fragmentation. Cells exposed to 10 µM DMNQ had no DNA fragmentation, whereas the DNA extracted from cells treated with 100 µM...
DMNQ exhibited high degrees of intranucleosomal DNA degradation, which appeared as a smear on the agarose gels (Fig. 6B). This is consistent with the observed accumulation of large amounts of single strand breaks.

Previous studies have shown that endonuclease-mediated DNA cleavage during apoptosis could be prevented by supplementing cells with polyamines (25) or by treatment with phorbol esters (41). When spermine (10 \( \mu \)M) was added to the incubation medium, DNA fragmentation by 30 \( \mu \)M DMNQ was partially prevented (Fig. 6C). In contrast, higher spermine concentrations were ineffective and in fact enhanced the non-enzymatic DNA damage (data not shown). Treatment with TPA (10 nM), which increased the intracellular spermine concentration (see above), was rather effective in preventing oligonucleosomal DNA fragmentation and apoptosis of cells exposed to 30 \( \mu \)M DMNQ (Fig. 6C). These pretreatments did not modify the DNA damage induced by 100 \( \mu \)M DMNQ, nor did they affect control cultures.

**Ca\(^{2+}\) Overload and Depletion of GSH, ATP, and NAD\(^{+}\) Precede RINm5F Cell Necrosis after Exposure to 100 \( \mu \)M DMNQ**—While treatment with either 10 or 30 \( \mu \)M DMNQ did not cause sustained modifications of the resting Ca\(^{2+}\) level (Table II), a progressive intracellular Ca\(^{2+}\) overload followed the exposure of cells to 100 \( \mu \)M DMNQ (Fig. 7A). The cytosolic free Ca\(^{2+}\) concentration increased up to 6 h, when a sizeable number of cells began to die. Incubation of RINm5F cells with 100 \( \mu \)M DMNQ also caused a progressive loss of GSH and ATP. Thiol levels rapidly declined, and, by 6 h, cells were virtually depleted of GSH (Fig. 7B). After 3 h, the ATP content of these cells was also reduced to about 15\% of that measured in controls (Fig. 7C). Under these conditions cell energy charge was well outside the range required for cell survival (normal range: 0.85—0.95; the energy charge was 0.91 in controls and 0.66 in cells exposed to 100 \( \mu \)M DMNQ for 3 h). Notably, both the glutathione level and cell energy charge were not significantly modified in cells exposed to either 10 or 30 \( \mu \)M DMNQ (Table II).

Rapid and marked depletion of the NAD\(^{+}\) pool also occurred in cells treated with 100 \( \mu \)M DMNQ (Fig. 7D). In contrast, as shown in Table II, NAD\(^{+}\) levels were maintained after treatment with 10 \( \mu \)M DMNQ. A moderate NAD\(^{+}\) decrease was also found in cells treated with 30 \( \mu \)M DMNQ, presumably because of the activation of DNA repair processes that require poly-ADP-ribosylation. Necrotic cell death (i.e. leakage of intracellular enzymes without DNA laddering and/or apoptotic body formation) followed the Ca\(^{2+}\) overload and the disruption of intracellular energy metabolism caused by the treatment with 100 \( \mu \)M DMNQ. As shown in Fig. 1B, after 3 h, cells had begun to release lactate dehydrogenase and by 9 h, only 40\% of the population had retained viability.

**DISCUSSION**

In the present study, we show that different prooxidant concentrations can alternatively stimulate growth or cause depletion of pancreatic RINm5F cells. Growth stimulation and the progression of the cell through the cell cycle are normally associated with the rapid induction of two short-lived enzymes involved in polyamine biosynthesis, ODC and SAMDC (42, 43). Protein kinase C activation mediates the induction of these
Cell Growth, Apoptosis, or Necrosis after Oxidative Stress

Figure 4. Intracellular polyamine content after exposure to DMNQ. Polyamines were separated by HPLC as described under “Experimental Procedures.” Panel A, 10 μM DMNQ: spermine (A—△); spermidine (□—○). Panels B and C, 30 μM DMNQ: spermine (A—△) and putrescine (□—○). Corresponding filled symbols show the level of each polyamine after preincubation with 10 nM TPA for 1 h. Polyamine concentrations in untreated cells were: putrescine, 103.3 ± 14.4 pmol × 10^6 cells, spermidine 953.7 ± 105.7 pmol × 10^6 cells, and spermine, 957.5 ± 109.8 pmol × 10^6 cells. Each of the data points is the mean ± S.D. of the average values from triplicate samples obtained in six separate experiments (*p < 0.05).

Enzymes (44, 45). In RINm5F cells exposed to low level oxidative stress, the involvement of protein kinase C in ODC induction is suggested by the effect of staurosporin, which abolished the growth stimulation elicited by 10 μM DMNQ and blocked ODC activation. The sensitivity to cycloheximide pretreatment also suggests that the increased enzyme activity be due to induction of new ODC molecules. The rapid increase of ODC activity correlated with the rise of intracellular polyamine levels up to 3 h after exposure to DMNQ; whereas the subsequent decrease in ODC activity was, most likely, the result of feedback inhibition by the elevated polyamine levels (42, 43).

When the DMNQ concentration was raised to 30 μM, cells died by apoptosis. Several observations have suggested a link between the mechanisms regulating cell proliferation and apoptosis. For example, deregulation of c-myc proto-oncogene expression or adenovirus E1A oncogene expression can stimulate both cell proliferation and apoptosis (46). Chromatin condensation and DNA degradation are considered the most typical hallmark of apoptotic cell death (40). While non-nuclear proteins may produce the characteristic nuclear changes of apoptosis in some systems (47), there is substantial evidence suggesting that modifications of the chromatin structure may be the primary signal for gene degradation and death in others (48). Previous studies in our laboratory have shown that DNA fragmentation and apoptotic body formation in thymocytes could be prevented by manipulating the intracellular content of polyamines (25). The effect of the polyamines, primarily spermine, correlated with their ability to promote chromatin compaction (24). Thus, it appeared conceivable that the suppression of apoptosis in thymocytes was due to their ability to reduce chromatin unfolding (25).

Polyamine depletion seems to play a role in chromatin degradation after exposure of RINm5F cells to 30 μM DMNQ, as suggested by the following observations: (i) apoptotic cells were
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Determinations of GSH, NAD+, ATP, ADP, and AMP levels were performed as indicated under “Experimental Procedures.” Values in resting, untreated RINm5F cells ranged between 0.85 and 0.93. Glutathione levels in untreated cells were: 9.13 ± 0.69 nmol/10^6 cells. Each value is the mean ± S.D. of three separate determinations.

TABLE II

|                  | Control                  | 10 μM          | 30 μM          |
|------------------|--------------------------|----------------|----------------|
|                  | 1 h  3 h  6 h            | 1 h  3 h  6 h  | 1 h  3 h  6 h  |
| Ca²⁺ (nM)        | 80 ± 11                  | 79 ± 15        | 78 ± 10        |
| GSH equivalents  |                          | 70 ± 20        | 60 ± 25        | 90 ± 21        | 81 ± 10        | 61 ± 25        | 90 ± 27        |
| (% of control)   |                          | 103 ± 1        | 95 ± 3         | 87 ± 9         | 105 ± 3        | 100 ± 7        | 87 ± 5         |
| Energy charge    | 0.87                     | 0.91           | 0.91           | 0.94           | 0.94           | 1.0            | 0.93           | 0.85           | 0.89           |
| NAD⁺ (nmol/10⁶ cells) | 1.1 ± 0.04              | 1.2 ± 0.1      | 1.2 ± 0.08     | 0.9 ± 0.2      | 0.8 ± 0.3      | 0.9 ± 0.09     | 0.8 ± 0.1      | 0.6 ± 0.15     | 0.7 ± 0.01     |

virtually depleted of putrescine and spermine; (ii) pretreatment with TPA before exposure to 30 μM DMNQ restored spermine content and prevented both DNA fragmentation and cell killing; (iii) addition of extracellular spermine reduced DNA fragmentation. The observation that treatment with the phorbol ester restored both the spermine level and the ability of the cells to grow supports the idea that DMNQ-induced depletion of intracellular polyamines was, at least in part, related to changes in protein kinase C activity. However, ODC is also sensitive to direct oxidation and it is possible that this may have contributed to the inactivation of the enzyme and its proteolytic cleavage (49). This latter assumption is supported by the finding that TPA pretreatment did not prevent the irreversible inhibition of ODC activity caused by DMNQ. It could then be questioned how TPA pretreatment would prevent spermine depletion without restoration of ODC activity. This can be explained by the finding that treatment with the phorbol ester prevented SAMDC inhibition by 30 μM DMNQ. SAMDC would provide decarboxylated S-adenosylmethionine for spermidine and spermine synthases (42, 43) resulting in increased spermidine and spermine levels, at the expense of putrescine. Our findings support this assumption, although we cannot exclude the involvement of additional alterations in polyamine metabolism.

Furthermore, exposure for 1 h to 30 μM DMNQ neither depleted intracellular polyamines nor did it trigger apoptosis. It is conceivable that reincubation in growth medium after 1 h may have supplemented mitogenic factors preventing polyamine depletion. In contrast, exposure of RINm5F cells to 30 μM DMNQ for 3 h followed by medium replenishment still caused cell death. This suggests that apoptosis was activated between 1 and 3 h, which coincides with the time when the intracellular polyamine level decreased. In this context, an increased intracellular polyamine content after mitogenic stimulation would provide protection against excessive chromatin exposure, whereas polyamine depletion may favor chromatin unfolding and cleavage during apoptosis.

DNA damage induced by 30 μM DMNQ appeared predominantly as oligonucleosomal fragmentation. Both 10 and 30 μM DMNQ stimulated ohdG formation and moderate DNA single

Fig. 6. DNA damage in RINm5F cells exposed to DMNQ. Panel A, DNA single strand breakage. Control (●—●); 1 μM DMNQ (○—○); 10 μM DMNQ (□—□); 30 μM DMNQ (■—■); 100 μM DMNQ (△—△). Results illustrate one experiment typical of three where the standard deviation never exceeded 5% of the mean values. Panel B, agarose gel electrophoresis of DNA extracted from RINm5F cells. DNA was separated and loaded on the gel as described under “Experimental Procedures.” Lane 1, control; lanes 2 and 3, 30 μM DMNQ, 3 and 6 h, respectively; lane 4, 10 μM DMNQ, 3 h; lane 5, 100 μM DMNQ, 3 h. Panel C, lane 1, 30 μM DMNQ, 3 h; lane 2, 30 μM DMNQ, 3 h; + 10 nm TPA; lane 3, 30 μM DMNQ, 3 h; + 10 μM spermine; lane 4, control.
strand breakage (cf. Table I and Fig. 6A). However, since both lethal and non-lethal DMNQ concentrations caused similar damage, it may be concluded that direct DNA damage by active oxygen species was not acutely lethal in this system. On the other hand, direct oxidative DNA damage, such as that induced by X-irradiation, can actually trigger apoptosis by inducing wild-type p53 gene expression (50, 51). This, in turn, results in the activation of growth arrest genes, cell cycle block, and death (52). Accordingly, alterations in the cell cycle clock due to increased demand for DNA repair, or to unbalanced mitogenic signals may favor gene exposure and chromatin degradation, which would be counteracted by a rise in the intracellular polyamine level. Following induction of the nitric oxide synthase activity and the subsequent nitric oxide accumulation (53), RINm5F cells express p53 protein2 and undergo apoptosis. It is conceivable that p53 expression may also take part in DMNQ-induced RINm5F apoptosis, although, at present, we have no conclusive evidence to support this assumption.

Finally, RINm5F cells rapidly died by necrosis when exposed to the highest prooxidant level. Under these conditions, cell death was preceded by GSH, ATP and NAD+ depletion, intracellular Ca2+ accumulation, and widespread DNA single strand breakage. It is therefore difficult to decide the most lethal event. Most likely, the recruitment of several catabolic events, with high degrees of oxidative stress, led to such swift cell disintegration that the selection of the organized, apoptotic program was prevented. Notably, while energy levels are believed to be preserved in apoptosis, necrosis is often associated with marked ATP depletion. Supporting this assumption is the observation that cell energy charge was preserved in cells exposed to 30 μM DMNQ (Table II). Similar findings have recently been obtained in experiments where neural cells underwent either apoptosis or necrosis following exposure to different glutamate concentrations. Therefore, one critical factor in deciding the type of death may be the energy level of the cell. Cells depleted of ATP and with a marked ion imbalance swell and rapidly lyse, whereas energy-dependent cell shrinkage and chromatin compaction would require a preserved energy.

2 M. Ankarcrona and P. Nicotera, unpublished observations.
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metabolism. This would explain, at least in part, the finding that oxidative stress can induce both necrosis and apoptosis (15 and this study) and that the cell death mode is dose-dependent. This possibility is currently under investigation in our laboratory.

REFERENCES
1. Crawford, D., Zhiden, I., Amstad, P., and Cerutti, P. (1988) Oncogene 3, 27–32
2. Birnbaim, H. (1992) Science 255, 1247–1249
3. Larsson, R., and Cerutti, P. (1986) J. Biol. Chem. 261, 17452–17458
4. Gopalakrishna, R., and Anderson, W. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 6768–6769
5. Kass, G. F. N., Duddy, S., and Orrenius, S. (1989) Biochem. J. 260, 499–507
6. Burdon, R., and Rice-Evans, C. (1989) Free Radic. Res. Commun. 6, 345–358
7. Murrell, G., Frances, M., and Bremley, L. (1990) Biochem. J. 265, 659–665
8. Chaudhuri, G., Clark, I. A., Hunt, N. H., Cowden, W. B., and Ceredig, R. (1986) J. Immunol. 137, 2646–2652
9. Harris, C. C. (1991) Cancer Res. 51, suppl. 5023–5044
10. Cerutti, P. A., and Trump, E. F. (1990) Cancer Cells (Cold Spring Harbor) 3, 1–7
11. Sies, H. (1985) in Oxidative Stress (Sies, H., ed) pp. 1–7, Academic Press Limited, London
12. Nicotera, P., Bellomo, G., and Orrenius, S. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 449–470
13. Orrenius, S., and Nicotera, P. (1987) Bull. Eur. Physiopathol. Respir. 23, 261–295
14. Malorni, W., Rivabene, R., Santini, M. T., Paradisi, S., Isosi, F., and Denelli, G. (1993) FEBS Lett. 336, 335–339
15. Kana, D. J., Sarafian, T. A., Anton, R., Hahn, H., Butler, J. R., Gralla, E., Selverstone, I., and Schimke, R. T., eds) pp. 6345–358
16. Warner, P., Eichner, R. D., Mullbacher, A., and Sjaurras, A. (1986) J. Biol. Chem. 261, 18405–18449
17. Hockeszber, D. M., Olsvai, Z. N., Yin, X., Millman, C., and Korsmeyer, S. J. (1993) Cell 75, 247–251
18. Williams, G. T., Smith, C. A., Spooner, E., Dexter T. M., and Taylor, D. R. (1990) Nature 343, 76–79
19. Kueng, A. L., Zettlberg, A., Sherwood, S., and Schimke, R. T. (1990) Cancer Res. 50, 7367–7373
20. Evans, G. L., Wylie, A. H., Gilbert, C. E., and Cook, A. (1987) FEBS Lett. 196, 9–12
21. Kana, D. J., Sarafian, F., Pellegrino, M. H. G., and Wilson, T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1120–1124
22. Snyder, E. D. (1989) Biochem. J. 260, 697–704
23. Tannen, R., Hartt, P., Nicotera, P., and Orrenius, S. (1991) Exp. Cell Res. 195, 323–329
24. Grasselli, B., Hartt, P., Nicotera, P., and Orrenius, S. (1991) Biochem. J. 260, 697–704
25. Brüne, B., Hartt, P., Nicotera, P., and Orrenius, S. (1991) Exp. Cell Res. 195, 323–329