# DNA Damage Associated with Ultrastructural Alterations in Rat Myocardium after Loud Noise Exposure

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**Noise exposure causes changes at different levels in human organs, particularly the cardiovascular system, where it is responsible for increasing heart rate, peripheral vascular resistance, and blood pressure. In this study, we evaluated the effect of noise exposure on DNA integrity and ultrastructure of rat cardiomyocytes. The exposure to loud noise (100 dBA) for 12 hr caused a significant increase of DNA damage, accompanied by swelling of mitochondrial membranes, dilution of the matrix, and cristolysis. These alterations were concomitant with increased *in situ* noradrenaline levels and utilization. Genetic and ultrastructural alterations did not decrease 24 hr after the cessation of the stimulus. An elevated oxyradical generation, possibly related to altered sympathetic innervation, is hypothesized as responsible for the induction and persistence of noise-induced cellular damage.** *Key words:* **comet assay, DNA damage, loud noise, mitochondria, myocardium, rat.** *Environ Health Perspect* **111:467–471 (2003). doi:10.1289/ehp.5847 available via** *http://dx.doi.org/* **[Online 6 December 2002]**

A large number of people are exposed to potentially hazardous noise levels during daily life, such as work environments, urban traffic, household appliances, and discos (Kaweckа-Jaszcz 1991; Lang et al. 1992). A variety of studies have suggested an association between noise exposure and the occurrence of disorders involving extra-auditory organs such as nervous, endocrine, and cardiovascular systems. Even brief noise exposure is known to increase heart rate (Linden et al. 1985) and peripheral vascular resistance (Bach et al. 1991), leading to a rise in blood pressure (Sawada 1993). On the other hand, experimental studies have demonstrated ultrastructural modifications in rat cardiomyocytes mainly involving mitochondria (Paparelli et al. 1995; Reuter 1966; Soldani et al. 1997; Yatani et al. 1999). These subcellular alterations are related to an imbalance in calcium homeostasis (Gesi et al. 2000), which is supposed to be sustained by increased catecholamine innervation (Breschi et al. 1994; Paparelli et al. 1992).

An increase in cytosolic calcium is also related to cell oxidative processes (Ermak and Davies 2002; Maciel et al. 2001); in line with this, noise exposure (110 dB for 1 hr) has been reported to increase reactive oxygen species (ROS) (Ohlemiller et al. 1999a). Mitochondrial permeability transition in various cells, including cardiomyocytes, is mediated by a concerted action between calcium and ROS (Castilho et al. 1995; Grijalba et al. 1999; Kowaltowski et al. 2001; Valle et al. 1993). For instance, calcium stimulates generation of ROS at specific sites of the inner mitochondrial membrane, where they attack protein thiols, thereby opening permeability transition pores (Fagian et al. 1990; Kowaltowski et al. 1996, 1998). One crucial effect of ROS is known to be the oxidative damage of nucleic acids (Cross et al. 1987; Lemasters et al. 1992), substantiated by single-strand breaks and inter/intrastrand cross-links (Caraceni et al. 1997). The persistence of ROS-mediated DNA alterations might lead to serious and long-lasting consequences, as suggested by the association between the persistence of ROS-mediated DNA alterations and mutagenic events (Emerit 1994). Despite several investigations focused on functional and morphologic effects even at the subcellular level, very little attention has been paid to the effects of noise on DNA integrity. Therefore, in the present study, we investigated whether levels of loud noise comparable with those present in modern daily life were able to produce DNA damage at the same doses and time intervals that trigger ultrastructural modifications to cardiomyocytes. At the same time, we sought to determine the occurrence of parallel increases in cardiac noradrenergic activity by measuring *in situ* noradrenaline (NA) turnover.

## Materials and Methods

***Animals.*** Male Wistar rats (weighing 200–250 g; Harlan Labs, San Pietro al Natisone, Udine, Italy) were used for the experiments. Animals were housed in the animal facility, fed *ad libitum*, and kept under closely controlled environmental conditions (12-hr light/dark cycle, lights on between 0700 and 1900 hr; room temperature, 21°C). The noise level in the rooms were animals were housed was about 10–30 dBA. Animals were treated in accordance with the *Guidelines for the Care and Use of Laboratory Animals* of the National Institutes of the Health (1996). All possible efforts were made to reduce animal suffering and to reduce the number of animals used.

***Experimental procedures.*** Noise was produced by two loudspeakers (15 W), installed at a distance of 40 cm at two opposite sides of the cage and driven by a white-noise generator emitting all the frequencies in the range 0–26 kHz. A precision sound level meter (Quest Electronic 215, Pisa, Italy) was used to set the intensity of sound to 100 dBA uniformly in the cage. During the experiment, the noise level peaked at 100 dBA immediately after the generator was switched on and lasted 12 hr. The level of 100 dBA was chosen as comparable with the noise frequently detected in discos and some industrial workplaces (Cohen et al. 1981). Control animals were sham exposed by placing them in the exposure chamber with the noise generator turned off. Animals were randomly assigned to experimental groups, each consisting of five specimens. Rats from group A were killed immediately after the noise stimulus, and their hearts were processed for comet assay, electron microscopy, and biochemical measurement (see below). Rats from group B were killed 24 hr after the end of noise exposure, and the myocardium was processed either for comet assay or for transmission electron microscopy. Groups C and D were used as controls, respectively, for groups A and B.

Experimental and control rats were sacrificed by decapitation to avoid the interference of deep anesthesia with DNA integrity and ultrastructure.

To ensure the reproducibility of findings, we repeated the experiment on two separated occasions. Sixteen animals from two separate experiments were used to evaluate DNA integrity soon after noise exposure, and eight animals from a single experiment were used to study the persistence/recovery of DNA damage 24 hr after the cessation of the stimulus.

***Assay of catecholamines.*** At the end of noise exposure, rats and their controls were sacrificed by decapitation. A small piece of the right atrium and ventricle from each rat was removed and processed for biochemical assay. The samples were sonicated in 0.6 mL of ice-cold 0.1 M perchloric acid, and an

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aliquot of homogenate (50 µL) was assayed for protein (Lowry et al. 1951). After centrifugation at 8,000 rpm for 10 min, 20 µL of the clear supernatant was injected into a high-performance liquid chromatography system to measure levels of NA and metabolites. Briefly, this consisted of a reverse-phase column (250 × 4.5 mm, C18, Beckman, Palo Alto, CA, USA) and two electrochemical detectors (analytical cell ESA 5011; Stagroma, Reinach, Switzerland). The potentials of the two electrodes were maintained at +0.35 and –0.35 V as previously described (Fornai et al. 1999). The signal was recorded at the reducing electrode. The mobile phase consisted of a citrate-phosphate buffer solution (0.04 M citric acid, 0.06 M sodium phosphate) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA), 0.6 mM sodium 1-heptanesulfonate, and 10% methanol. The flow rate was set at 1 mL/min. A standard curve was prepared using known amounts of NA and metabolites (Sigma Chemical Co., St. Louis, MO, USA), dissolved in perchloric acid (0.1 M) containing a constant amount (10 pg/µL) of the internal standard [dihydroxybenzylamine (DBA)], as used for the tissue samples. The standard curve for each compound was calculated using regression analysis of the ratio of the peak areas (compound area/DBA area) for various concentrations of each compound.

***Light microscopy.*** To check for potential occurrence of myocardial cell death, tissue samples from each heart were taken and processed using routine histologic procedures. Briefly, sections 8–10 µm thick were cut with a microtome and stained with hematoxylin-eosin and toluidine blue. No myocardial cell death was observed.

***Transmission electron microscopy.*** After sacrifice, the still-beating heart was dissected and 0.5 $mm^3$ samples from the right atrium (at the lateral side of the vena cava opening) and the right ventricle (near the apex) were immersed in 3% glutaraldehyde fixing solution for 60 min. Time from sacrifice to fixation was 60 ± 20 sec for all animals. Fixation by immersion was preferred to perfusion technique in order to obtain living cells from the same heart to be used for DNA damage evaluation as well. Moreover, no significant differences were previously observed in the subcellular structure of rat cardiomyocytes fixed by both immersion and perfusion techniques (Lenzi et al. 1998). Specimens were postfixed for 2 hr at 4°C in 1% buffered $OsO_4$, dehydrated in ethanol, and embedded in Epon-Araldite. Sections were then observed under a JEOL JEM 100 SX transmission electron microscope (JEOL, Tokyo, Japan). For each rat, two tissue blocks were chosen at random and 10 electron micrographs were examined at a final magnification of 10,000×. At first, the extent of the damage was analyzed observing the general ultrastructure of the cell. The percentage of altered mitochondria was recorded to give a quantitative estimation of ultrastructural damage. To avoid experimental bias concerning nonspecific ultrastructural alterations induced by the fixing procedure, we followed a validated schedule for electron microscopy as previously reported (Lenzi et al. 1998; Marino et al. 1983; Soldani et al. 1997; Tomanek and Karlsson 1973).

***Evaluation of DNA damage.*** DNA integrity was evaluated by the use of alkaline single-cell gel electrophoresis or comet assay, according to Singh et al. (1988), with minor modifications.

Briefly, isolated cells are embedded in agarose on a microscope slide, lysed with detergent, and treated with high salt. Any breaks present in the DNA cause the supercoiling to relax locally, and negatively charged loops of DNA are then free to extend and migrate in the electric field toward the anode as a "comet tail."

After sacrifice, separate specimens from atria and ventricles were washed in cold phosphate-buffered saline and then placed in 1 mL of chilled mincing solution ($Ca^{2+}$, $Mg^{2+}$-free Hank's balanced salt solution, 20 mM $Na_2EDTA$, 10% dimethyl sulfoxide, pH 7.5). The tissue was cut in small pieces by scissors. After 15 min, the supernatant was centrifuged for 10 min at 1,000 rpm. The assessment of cell viability on individual cells was not possible in these conditions because during mincing and dissociation processes, cell membrane was disrupted (Singh et al. 1995). The pellet obtained was mixed with 75 µL of agarose [0.5% low–melting-point agarose (LMPA) prepared in $Ca^{2+}$, $Mg^{2+}$-free phosphate-buffered saline) and layered on conventional slides, predipped in 1% normal–melting-point agarose (Klaude et al. 1996). Then, a third layer of 85 µL LMPA was added. Slides were immersed in ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM $Na_2EDTA$, 10 mM Tris-HCl, 1% Triton X-100, and 10% dimethyl sulfoxide, pH 10) to lyse the cells and allow DNA unfolding. After 1 hr at 4°C in the dark, slides were covered with an alkaline solution (1 mM $Na_2EDTA$, 300 mM NaOH, pH > 13) in a horizontal electrophoresis unit for 20 min to allow DNA unwinding and expression of alkali-labile sites. Slides successively underwent electrophoresis (25 V, 300 mA, 20 min) in an ice-cold bath. Next, slides were washed gently with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) to remove alkali and detergents, dipped in 100% cold methanol, and dried. After drying, slides were stained with 100 µL of ethidium bromide (2 µL/mL). All the steps described above were conducted under yellow light or in the dark, to prevent nonspecific DNA damage. DNA migration is proportional to the level of DNA damage (Figure 1).

Because of the very low molecular weight of the DNA in terminal apoptotic cells, the DNA of these cells may be lost from the gels under the typical electrophoretic conditions used. Therefore, the diffusion assay was used to assess whether apoptosis was induced by noise exposure in heart cells. According to this technique, slides were removed from the lysing solution, neutralized with Tris-HCl, stained with ethidium bromide, and observed under a fluorescence microscope to evaluate the frequency of cells with an extremely diffuse staining pattern. In nonelectrophoretic conditions, apoptotic cells were identified by the presence of highly dispersed DNA giving rise to a characteristic halo around the nucleus (Tice et al. 2000; Vasquez and Tice 1997). At least 100 cells per data point were scored and the percentage of apoptotic cells evaluated.

***Statistical analysis.*** For biochemical assay, results from control and noise-exposed rats are expressed as the mean ± SE of values obtained from groups of five rats. The effects of noise stress on NA and metabolites were compared using one-way analysis of variance (ANOVA) with Sheffè's *post hoc* analysis.

Because atrium and ventricle showed different morphologic responses to noise, they were analyzed separately. Results were calculated by counting the number of altered

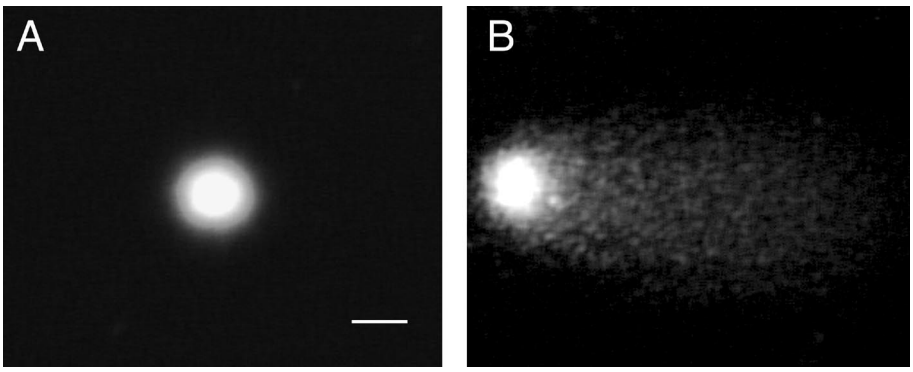


**Figure 1.** Atrial cardiomyocyte nuclei after comet assay. (*A*) Nucleus with undamaged DNA. (*B*) Nucleus with about 50% of DNA in the tail. Bar = 20 µm.

mitochondria and are expressed as a percentage of the mean ± SE of values obtained from groups of five rats. Differences between groups were compared using one-way ANOVA with Sheffè's *post hoc* analysis. For each comparison, the null hypothesis was rejected when $p < 0.01$.

For the comet assay, nuclei were individually observed under a fluorescence microscope (200×), and an image analyzer (Komet, version 4; Kinetic Imaging Ltd., Bromborough, UK) was used to evaluate the percentage of DNA migrating in the tail of at least 25 cells per slide. Slides were coded and scored blindly to avoid risk for bias. Four parallel tests were performed per experimental point for a total of at least 100 cells, and the mean was calculated. Data were analyzed using multifactor analysis of variance (MANOVA), performed on log-transformed data in order to approach the normal distribution. MANOVA was used to assess the significance of factor effects, such as animals, cultures, and doses. For statistical analysis, the software SGWIN (Windows 98) was used.

## Results

***DNA integrity.*** The effects of loud noise on the presence of DNA damage in rat cardiomyocytes, evaluated as percentage of DNA in the tail after electrophoresis, are shown in Figure 2A. The exposure of rats to noise for 12 hr caused a significant increase of DNA migration ($p < 0.001$) in atrial and ventricle myocardium compared with controls. Almost unchanged patterns of DNA migration were observed 24 hr after the cessation of the stimulus, suggesting the absence of recovery (Figure 2A). Noise-induced DNA damage was higher in the ventricle than in the atrium ($p < 0.01$), both soon after the exposure and 24 hr later. This suggests a differential susceptibility of these myocardial regions to noise.

The results of the diffusion assay did not reveal the occurrence of apoptotic cells either in exposed or control animals, at any time interval, confirming the absence of cell death found at light microscopy. This finding allows us to exclude the possibility that the amount of strand breaks observed in the present study might be due to the nonspecific loss of DNA integrity related to cell death processes, supporting the genotoxic effect of loud noise.

***Electron microscopy.*** **Right atrium.** The subcellular structure of controls (groups C and D) exhibited a typical pattern: sarcoplasmic reticulum, atrial granules, and intermyofibrillary mitochondria with dense matrix and well-arranged cristae (Figure 3A). Rats sacrificed immediately after exposure to loud noise (group A) showed significant ultrastructural alterations at the mitochondrial level, consisting of swelling of the membranes, dilution of the matrix, and cristolysis (Figures 2B, 3B–D). A similar effect was still observed in the atrial tissue of group B rats sacrificed 24 hr after the end of noise exposure (Figure 2B). No ultrastructural evidence of apoptosis was observed, such as chromatin condensation or looping of the nuclear envelope, confirming diffusion assay data.

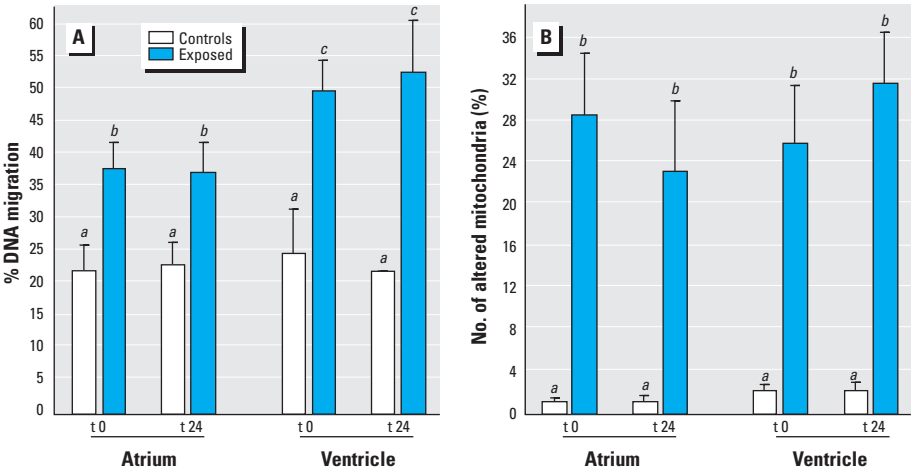


**Figure 2.** DNA damage (*A*) and mitochondrial alterations (*B*) induced in the rat myocardium by loud noise exposure. Data are expressed as mean values of (*A*) DNA migrations or (*B*) percentage of altered mitochondria in both atrial and ventricular tissue after 12 hr of noise exposure. Rats were killed either immediately (t 0) or 24 hr (t 24) after the end of noise exposure. The percentage of DNA fragmentation (*A*) or altered mitochondria (*B*) in nonexposed rats is included for comparison. Each group was composed of five rats. Mitochondria were classified as altered when either matrix dilution or cristolysis was present, as shown in Figure 3. Different letters indicate significant differences among means ($p < 0.01$).

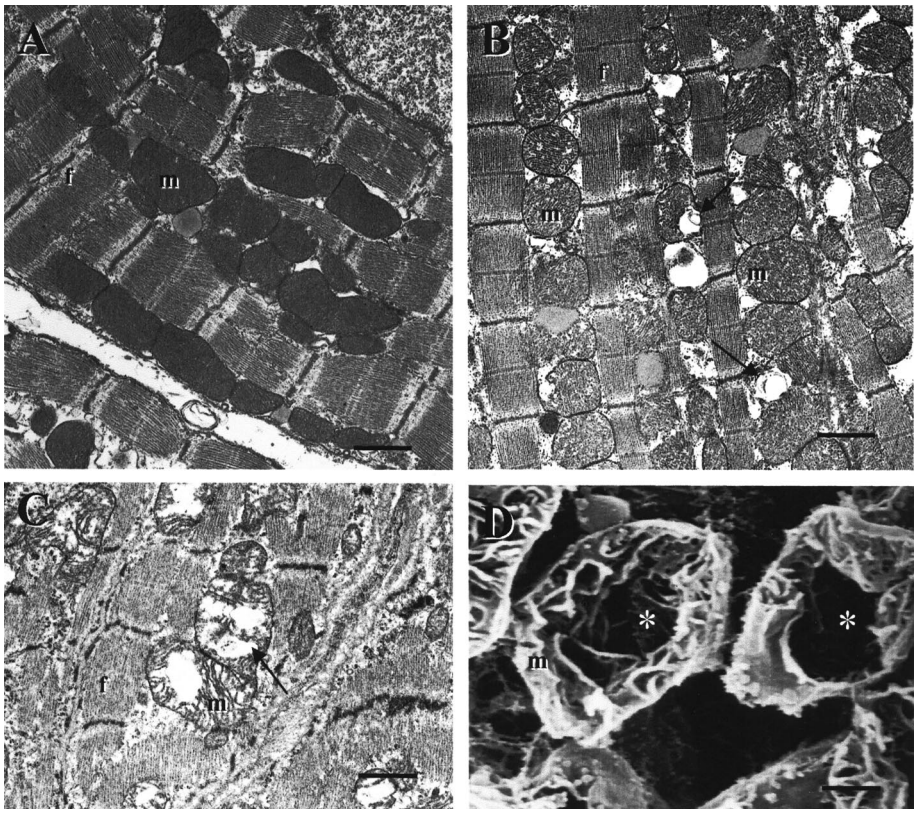


**Figure 3.** Effects of loud noise on the ultrastructure of atrium. Abbreviations: m, mitochondria; f, myofibrils. Arrows indicate altered mitochondria. (*A*) Transmission electron micrograph from myocardium of control rat. Mitochondria possess a well-preserved structure. Scale bar, 4 µm. (*B*) Cardiomyocyte from 12-hr noise-exposed rat. Altered mitochondria with dilution of the matrix and cristolysis are evident. Scale bar, 4 µm. (*C*) High magnification of exposed rat mitochondria showing marked cristolysis and dilution of the matrix. Scale bar, 7 µm. (*D*) High-magnification scanning electron micrograph of exposed rat showing mitochondria with swollen empty space (*) in which some cristae are seen. Bar = 20 µm.

**Table 1.** Catecholamine content in the myocardial tissue from control rats and rats exposed for 12 hr to noise.

| | Control | Noise exposure |
|---|---|---|
| Atrium | | |
| NA | 1.86 ± 0.18 | 4.78 ± 0.58* |
| DOMA | 0.30 ± 0.11 | 0.80 ± 0.13 |
| VMA | 61.17 ± 3.92 | 147.55 ± 7.21* |
| Ventricle | | |
| NA | 4.02 ± 0.78 | 12.53 ± 2.12* |
| DOMA | 0.9 ± 0.23 | 1.56 ± 0.69 |
| VMA | 68.18 ± 2.36 | 152.36 ± 3.25* |

Levels of NA, and its metabolites vanilmandelic acid (VMA) and dihydroxymandelic acid (DOMA) levels are the mean values ± SE of 5 animals per group.
*$p < 0.05$ compared with control.

**Right ventricle.** The ventricles from controls (groups C and D) showed a normal ultrastructure, with intermyofibrillary mitochondria provided with a dense matrix (data not shown). The cardiomyocytes of noise-exposed rats in groups A and B revealed significant mitochondrial alterations similar to those described in atrial tissue (Figure 2B).

Although the morphology of the mitochondria was altered by loud noise in exposed specimens, no changes in the total number of mitochondria were detected in comparison with control tissue. Particularly, in control rats the number was 0.21 ± 0.03 mitochondria/$\mu m^2$ and 0.24 ± 0.02 mitochondria/$\mu m^2$ for atrium and ventricle, respectively.

***Assay of catecholamines.*** Measurement of NA levels in specific areas of myocardial atrium and ventricle demonstrated the presence of higher amounts of this catecholamine in control tissue from the ventricle compared with that from the atrium (Table 1). Extending what was previously reported on the basis of histofluorescence qualitative measurements (Paparelli et al. 1992), the amount of NA and its metabolites dihydroxymandelic acid and vanilmandelic acid in myocardial tissue after noise exposure indicated that loud noise increases NA levels and NA utilization. Increase in extracellular metabolites significantly exceeded increases in NA levels, leading to a significant rise in the turnover index (ratio between extracellular metabolites and NA).

## Discussion

This study demonstrates that loud noise exposure produces in myocardial cells both ultrastructural and molecular alterations in the absence of myocardial cell death.

In particular, the major finding consists of demonstrating for the first time that 12 hr of exposure to loud noise was able to induce genetic damage. The amount of DNA damage induced by acoustic stress did not decline rapidly, persisting almost unchanged 24 hr after the cessation of the stimulus. We can exclude the possibility that the electrophoretic migration of DNA was caused by cell-death–associated fragmentation; indeed, diffusion assays revealed a negligible occurrence of apoptotic cells, and light and electron microscopy did not reveal any necrotic and/or apoptotic event. Concerning the persistence of genetic damage, it is noteworthy that DNA single-strand breaks are usually repaired within 15 min and that DNA double-strand breaks are repaired within 2 hr (Plappert et al. 1997; Vijayalaxmi et al. 1993). Thus, the persistence of a clastogenic agent, produced as a consequence of noise exposure, is to be supposed. In addition, the generation of alkali-labile abasic sites might account for at least part of the persistent DNA damage in heart cells. In fact, alkali-labile sites, which are also detected by the alkaline comet assay, are known to persist longer than strand breaks and might also represent intermediates in the repair of lesions other than frank breaks, such as oxidized bases. These molecular effects are accompanied by morphologic alterations in the mitochondria that can be summarized as dilution of the matrix and cristolysis. Similar to the persistence of DNA fragmentation, in the present study we found that subcellular alterations were not reduced at 24 hr after the end of noise exposure, suggesting either the severity of the morphologic damage or the persistence of an altered cellular metabolism. Indeed, both hypotheses might coexist; nonetheless, the similar persistence of DNA damage as measured by the comet assay at 24 hr after the end of the exposure suggests that an ongoing pathologic effect in the myocardial cell is still working. The subcellular alterations observed with electron microscopy show that damage measured at 24 hr after the cessation of the stimulus was slightly higher compared with that found immediately after noise exposure. Interestingly, both DNA and mitochondrial damage, apart from having a similar time course, showed a comparable topographical pattern, the ventricle being the myocardial region that is more sensitive to the consequences of loud noise.

The persistence of a pathologic agent needs to be deeply investigated and calls for prolonged time-course studies to evaluate the potential recovery or even a further worsening at longer time intervals. In particular, here we found that noise exposure produces a significant increase of NA release as measured by myocardial levels of NA and metabolites. This latter finding lends substance to previous observations obtained in noise-exposed rats showing an increased fluorescence in the myocardial tissue as revealed by the histochemical technique of Falck-Hillarp (Paparelli et al. 1992). These original findings did not address the functional relevance of an increased myocardial histofluorescence, leaving open the question of whether this was accompanied by increased NA release. The present findings provide evidence of increased myocardial NA turnover during loud noise exposure. Increased sympathetic innervation is considered the primary event leading to the noise-induced calcium entry that we recently demonstrated (Gesi et al. 2000; Salvetti et al. 2000). Confirming this chain of events, we recently found a topographical correspondence between altered mitochondrial areas and localization of calcium deposits (Gesi et al. 2000). Altered cytosolic calcium influx might trigger oxidative processes (Ermak and Davies 2002; Kowaltowski et al. 1998; Maciel et al. 2001) and subsequent DNA damage. On the other hand, ROS have been implicated in a growing number of chronic disease conditions, as well as cell damage induced by environmental pollutants and physical agents (Collins et al. 1998; Emerit 1994; Sarker et al. 1995). In this respect, considerable evidence suggests that ROS are also responsible for noise-induced alterations (Ohlemiller et al. 1999a). These authors also reported a 4-fold increase, and a persistence after exposure, of the highly toxic hydroxyl radical in mice subjected to loud noise (Ohlemiller et al. 1999b).

It is likely that a calcium-related event (i.e., activation of a specific metabolic pathway or transduction mechanisms) might lead to a long-lasting process responsible for sustaining DNA damage even when noise exposure is ceased. A similar phenomenon may underlie the lack of recovery from ultrastructural changes. In this respect, it might be hypothesized that genetic instability may be related to the development of cardiovascular diseases (Andreassi et al. 2000).

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