HEALTH PSYCHOLOGY, 1985, *4* (5) 403–412
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EMPIRICAL CONTRIBUTIONS

Effects of Stress on Methyltransferase Synthesis: An Important DNA Repair Enzyme

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The enhancement of tumor development following acute stress has been demonstrated in some animal studies. This study was designed to explore mechanisms that would account in part for the relationship between stress and tumor development at the level of DNA repair, using a rat model. Forty-four rats were given the carcinogen dimethylnitrosamine in their drinking water, and half were randomly assigned to a rotational stress condition. The levels of methyltransferase, a DNA repair enzyme induced in response to carcinogen damage, were significantly lower in spleens from the stressed animals. These data suggest that stress may impair DNA repair.

Exposure to low-level carcinogens is a commonplace daily event. For example, nitrates are chemical carcinogens found in many processed meats, some American beers, and spinach. The benzopyrenes, another group of chemical

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carcinogens, are found in smoke and soot, and aflatoxin is the carcinogenic product of a mold associated with peanuts and certain grains (Miller, 1978).

Most carcinogens appear to induce cancer by damaging the DNA in cells. If the damaged DNA in a cell is not properly repaired, a mutant cell may develop and proliferate (Setlow, 1978). Fortunately, most carcinogen exposure is at low levels and for limited periods of time, and most DNA changes are probably not cancerous.

Moreover, the body has a hierarchy of defenses for dealing with carcinogen exposure (Fox, 1981). At the first level are enzymes that destroy chemical carcinogens. The second level involves the processes used to repair damaged DNA. The third level is the immune system's destruction of mutant cells; in this regard, natural killer (NK) cell activity is thought to be an important defense against tumor cells.

The second level, the repair of damaged DNA, is particularly critical in humans. There is very good evidence that faulty DNA repair leads to an increased incidence of cancer (e.g., Setlow, 1978; Takabe, Yagi, & Satoh, 1983). Even small deviations from normal DNA repair levels may have critical biological consequences (Setlow, 1983).

In an earlier work from our laboratory, we found distress-related differences in DNA repair. There was significantly poorer repair of DNA damaged by X-irradiation in leukocytes obtained from new nonpsychotic, nonmedicated psychiatric admissions, compared to age- and sex-matched Red Cross blood donors; although the DNA from the blood donors was fully repaired at the 5-hr end point, the DNA from the inpatients was repaired to only 92% of the baseline values. In addition, within the psychiatric patient group, leukocytes from the more depressed inpatients had significantly poorer DNA repair than leukocytes from the less depressed patients (Kiecolt-Glaser, Stephens, Lipetz, Speicher, & Glaser, in press).

These data show a significant association between higher levels of distress and impaired DNA repair. However, it is possible that both distress and poorer DNA repair could be a function of another variable (e.g., some common genetic predisposition). Most of the DNA repair enzymes (i.e., N-glycosolases and endonucleases) are constitutively produced by cells and thus are less likely to be affected by outside factors such as stress. Exceptions to this are the transferases responsible for repairing O^6-alkylguanine. The alkyl transferases are consumed in the repair process and act as a regulator of their own synthesis. For this reason, we decided to examine the repair enzyme O^6-methylguanine DNA methyltransferase (Swenberg, Bedell, Billings, Umbenhauer, & Pegg, 1982). This enzyme is inducible in response to damage from carcinogens and was therefore considered most likely to be affected by stress (Lindamood, Bedell, Billings, Dyroff, & Swenberg, 1984). The carcinogen of choice was dimethylnitrosamine (DMN) because it has been shown that O^6-methyltransferase can be induced by DMN in liver and kidney tissue (Greim, Jung, Kramer, Marquardt, & Oesch, 1984).

A number of studies have shown that tumor development and growth in rodents are enhanced under certain stressful conditions (Ader, 1981). Of particular relevance to the present study are data from Riley (1981) showing enhanced tumor induction and growth following intermittent rotational stress. Rotational stress was also associated with increased plasma concentrations of adrenal corticoids and other hormones which appeared to depress cell-mediated immunocompetence.

The present study was designed to test the hypothesis that stress per se would result in an impairment in an important DNA repair enzyme. This study used an animal model and employed slow rotation of the home cage as a stressor (Riley, 1981).

METHOD

Animals

Forty-nine experimentally naive female Sprague-Dawley rats (90–120 days old) were used. The rats were obtained from university breeding facilities and were adapted to the experimental environment for 2 weeks prior to behavioral testing. Two animals were housed to a cage in a biohazard protected room. All animals were handled one time per week, during which time the bedding was changed. A diurnal cycle was maintained, with lights on at 7:00 a.m. and off at 7:00 p.m. The room is designed to have 15 complete changes of air per hr, 24 hrs a day.

Rotational Stress Procedures

Rotational stress is a relatively benign procedure that produces adrenal cortical activation without subjecting the animal to unnecessarily harsh treatment or depriving it of food and water (Riley, 1981). A modified phonograph table allowed rotation of the animal's home cage, and a slow rotational speed (45 rpm) permitted the animals to continue to move about the cage, eat, and drink. The rotation is not a centrifugal force, but rather induces a mild spatial disorientation.

Animals were randomly assigned to one of three experimental groups: (a) stress/carcinogen ($n = 22$); (b) nonstress/carcinogen ($n = 22$); or (c) nonstress/noncarcinogen ($n = 5$). The small nonstressed noncarcinogen group was included to evaluate enzyme synthesis in the absence of the carcinogen; we did not include a stressed noncarcinogen group in the design because differences in methyltransferase activity as a function of stress should not occur in the absence of the carcinogen.

Four 24-hr periods of rotational stress were given during the experimental phase of the study. Animals were given their first 24-hr period of stress the

day after they had initially received the carcinogen and 6, 10, and 14 days after carcinogen exposure began. Each 24-hr period involved cycles of 10-min of rotation, followed by a 50-min rest. On day 16 of carcinogen administration, animals were decapitated. Spleens were dissected and immediately frozen in liquid nitrogen. The order of euthanization was matched so that one animal from each experimental group was decapitated at the same time.

Exposure to Carcinogen

Carcinogen-treated animals were exposed to 50 parts per million (ppm) DMN in their drinking water for 16 days (Lindamood et al., 1984). Based on the average fluid intake per rat, this represents approximately 3.75 mg/kg/day of carcinogen; there were no reliable differences among the three groups in fluid intake.

Preparation of DNA Substrate

In preparation for the methyltransferase assay, a DNA substrate was prepared (Lindamood et al., 1984). Calf thymus DNA was treated with tritiated N-methyl-N-nitrosourea (MNU) at 37°C for 2 hrs in order to produce alkylation. The DNA was then dialyzed against buffer to remove the MNU. The alkylated calf thymus was heated for 16 hrs at 80°C to remove the N-alkylations. The preparations were dialyzed again three times at 4°C overnight against 800 ml of assay buffer containing 75 mM NaCl, 50 mM tris-HCl, 0.1 mM Na-2-EDTA, and 1 mM dithiothreitol. The percentage of alkylation at the O^6 position of guanine was determined by high performance liquid chromatography and was found to be between 85%-90%.

Methyltransferase Assay

The spleens that were removed from the rats were minced, homogenized, and assayed for methyltransferase as previously described (Krokan, Haugen, Myrnes, & Guddel, 1983). The suspensions were centrifuged for 15 min at 7,500 rpm, using a Beckman J2-21 centrifuge at 4°C. The soluble protein fraction (supernatant) was assayed for total protein using the BioRad protein assay (Bradford, 1976). The supernatants were aliquoted into tubes at 0.5, 1.0, or 1.5 mg of protein. The volume of each preparation was adjusted to 120 μl using assay buffer. Incubation of methyltransferase with O^6-methylguanine containing DNA results in the transfer of a methyl group from the guanine to a sulfhydryl group of the protein. Solubilization of the protein with proteinase K releases radiolabeled methyl groups into the supernatant. The aliquots of protein were incubated with calf thymus DNA substrate (2000 DPM) for 40 min at 37°C. After the 40-min incubation, 7 μl of 10

mg/ml proteinase K and 5 μl of 25% sodium dodecyl sulfate (SDS) (final concentration 1%) were added. Paired controls for three spleen preparations at each protein concentration were used in which equivalent amounts of protein and substrate were added, but not proteinase K or SDS. Twelve μl of assay buffer were added to the controls. The reaction mixture was incubated for an additional hour at 37°C, and the DNA was then precipitated by the addition of 27 μl of 1.04 M NaCl, 25 μl of carrier DNA (calf thymus DNA, 2 mg/ml), and 444 μl of 1.115% trichloroacetic acid in 80% ethanol. The samples were incubated at −20°C for at least 30 min, centrifuged at 15,000 rpm for 12 min at 4°C, and the level of radioactivity (DPM) in the supernatant was determined by liquid scintillation using a Beckman LS-1800 scintillation counter. The activity was determined by the following formula:

$$\frac{\text{Sample DPM - Background DPM}}{1.188 \times 10^{13}\ \text{DPM/pmol (based on the specific activity of the substrate, MNU)}}$$

All values were adjusted to pmole/mg protein.

RESULTS

Relationship of Methyltransferae Activity to Level of Protein

In order to determine if the concentrations of protein used in the assays were in the linear range of enzyme activity, correlations were calculated between methyltransferase activity and the three protein concentrations. The protein preparations used to determine this range were obtained from the spleens of the nonstressed carcinogen-treated group. We found that the protein concentrations utilized were within the linear range of enzyme activity, with a correlation of 0.987, as shown in Fig. 1. This was confirmed when the adjusted enzyme activity levels (in pmoles/mg) were statistically analyzed using repeated ANOVA; there were no significant differences among the three protein concentrations, as discussed later. Similar results were obtained when protein preparations from spleens of animals from the other groups were used.

Methyltransferae Activity in the Spleens of Test Animals

To test the effects of the carcinogen alone on enzyme synthesis, spleens from each of the two groups of carcinogen-fed rats were compared to the control group using the Dunnett test (Winer, 1962). The level of methyltransferase in the spleens from the carcinogen-fed rats differed significantly

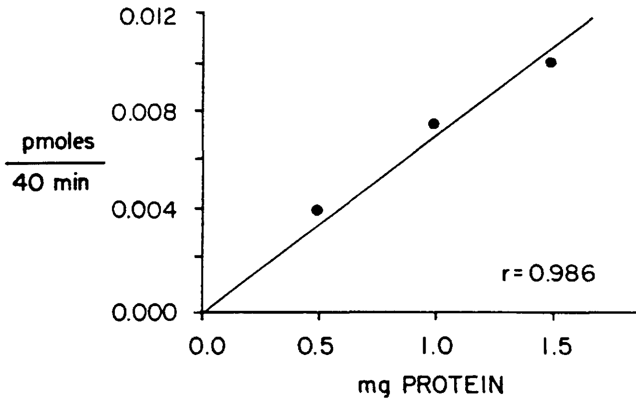

FIG. 1 Relationship of methyltransferase activity to protein concentration in spleens from rats from the nonstressed carcinogen-treated group.

from those in the control group, both at $p < .01$, indicating suppression of repair (methyltransferase activity) by the carcinogen itself.

Using enzyme activity expressed as pmoles of enzyme/mg protein, data from the three normalized protein concentrations for the carcinogen-fed stressed and carcinogen-fed nonstressed rats were compared in a 3 × 2 repeated analysis of variance (ANOVA) with one within-subjects variable (the three protein concentrations) and one between-subjects variable (carcinogen-treated stressed vs. carcinogen-treated nonstressed animals). There was a significant difference between the methyltransferase activity in spleens from stressed animals compared to the nonstressed group, $F(1,42) = 4.11$, $p < .05$, indicating a suppression of repair associated with methyltransferase by the stressor. There were no significant differences among the three protein concentrations, $F(2,84) = 3.00, p < .11$, and the interaction between the two independent variables was not significant, $F < 1$. These data are shown in Table 1.

DISCUSSION

We found significantly less methyltransferase activity in the spleens of rats exposed to rotational stress than in the nonstressed rats. Because methyltransferase is an important enzyme for DNA repair, the lower levels of the enzyme associated with stress provide evidence of one pathway through which stress could impair or suppress the repair of carcinogen-induced damage to DNA, potentially altering the efficacy and/or speed of the repair process. The stress-related methyltransferase differences are not large; however, Setlow (1983) has reviewed data suggesting that even small deviations from

the "normal" DNA repair process may produce very significant increases in cancer incidence under some conditions. It is not known what actual minimal dosage of a carcinogen is sufficient to produce cancer in humans.

Only one biohazard protected room was available for this study. As a consequence, both stressed and nonstressed rats were housed in the same room, and the rotational stress procedures were also carried out in this room. Under these conditions, the nonstressed rodents were subjected to noise, pheromones, and ultrasound distress signals from the stressed group; therefore, they may have been stressed, albeit at a much lower level, at the same time (Borysenko & Borysenko, 1982). Hence, these data may actually underestimate the effects of stress on methyltransferase synthesis.

The levels of methyltransferase in the carcinogen-treated groups were lower than in the smaller group, which was not given the carcinogen, suggesting suppression of repair by the carcinogen. DMN has been shown to suppress some aspects of immune function (Thomas et al., 1985). Because the spleen is a lymphoid organ, it is quite possible that it has similar adverse effects on splenic lymphocytes. The decrease in O^6-methyltransferase activity in the carginogen-treated groups probably was not due to cytotoxicity because we performed viability cell counts on circulating peripheral blood lymphocytes (PBL) (data not shown); more than 90% of the cells were viable in all groups. If in vivo levels of DMN achieved in the rats were toxic to splenic lymphocytes, we should have observed concomitant toxicity with the PBLs.

The data in this study are consistent with previously discussed differences in DNA repair found between high and low distressed psychiatric patients (Kiecolt-Glaser, Stephens et al., in press). Putting the two studies together, it appears that stress or distress may have measurable negative consequences at the molecular level, with clear relevance for carcinogenesis.

As mentioned earlier, a number of studies have shown that tumor development and growth are enhanced under certain stressful conditions (Ader, 1981). Additional studies also document the immunosuppressive effects of certain stressors. Convergent data from rodents and humans show stress-related decreases in natural killer (NK) cell activity, which is thought to be of

TABLE 1
Mean (± S.E.M.) Methyltransferase Activity in Spleens

	Carcinogen Treated		*Noncarcinogen Treated*
Protein (in mg)	*Stressed (n = 22)*	*Nonstressed (n = 22)*	*Nonstressed (n = 5)*
0.5	0.0086 (0.001)	0.0120 (0.001)	0.0290 (0.002)
1.0	0.0089 (0.001)	0.0119 (0.001)	0.0210 (0.002)
1.5	0.0090 (0.001)	0.0132 (0.001)	0.0180 (0.001)

Note. Activity expressed as pmole enzyme/mg protein.

particular importance because of the antitumor and antiviral functions of NK cells (Herberman, 1982; Kiecolt-Glaser, Garner et al., 1984; Kiecolt-Glaser, Glaser et al., in press; Kiecolt-Glaser et al., 1985; Shavit, Lewis, Terman, Gale, & Liebeskind, 1984). It is now known that a number of other immune functions are also affected by relatively commonplace stressful events (Glaser, Kiecolt-Glaser, Speicher, & Holliday, in press; Jemmott et al., 1983; Kiecolt-Glaser, Speicher, Holliday, & Glaser, 1984). These DNA repair data are particularly important in the context of stress-related declines in NK cell activity; they show that stress or distress could contribute directly to carcinogenesis through impaired DNA repair, as well as indirectly by adversely affecting immune surveillance and destruction of tumor cells.

It is not clear that such stressors as periodic rotation parallel the physiological effects of psychological or social stress in humans. Although such "physical" stressors clearly promote tumor development and growth in rodents under certain conditions (Borysenko & Borysenko, 1982), humans are rarely exposed to comparable conditions. If, however, higher levels of distress have some causal association with carcinogenesis, then more distressed populations should have a higher cancer risk.

In fact, there are epidemiological data which link distress and cancer risk. For example, in one 17-year study of over 2,000 nonpsychiatric men, depression was related to significantly greater cancer mortality across cancer sites, after correction for a number of relevant risk factors (Shekelle et al., 1981). Moreover, other data suggest that psychiatric patients have greater cancer mortality than nonpsychiatric populations (Fox, 1978). The data in this study provide evidence for one physiological pathway through which stress could directly influence the development of malignant disease.

ACKNOWLEDGMENTS

This study was supported in part by the Roessler Scholarship Fund, by the College of Medicine, Ohio State University, and by the Comprehensive Cancer Center Core Grant No. CA-16058. We thank Julie C. Stout and Cynthia Beaulieu for excellent technical assistance.

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