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Poly(ADP-ribosylation) of Histones in Intact Human Keratinocytes†

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ABSTRACT: The poly(ADP-ribosylation) of chromosomal proteins is an epigenetic consequence of clastogenic DNA damaging agents which affects chromatin structure and function. We studied the poly(ADP-ribosylation) of the major classes of histones in response to DNA breakage induced by an extracellular burst of active oxygen (AO) or the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in the immortalized human keratinocytes HaCaT using a combination of affinity chromatography on phenylboronate resin and immunoblotting with polyclonal antibodies against histones H1, H2B, H2A, H3, and H4. The following findings characterized the poly(ADPR) reaction: (1) pretreatment of nuclear extracts with snake venom phosphodiesterase which removes poly(ADPR) chains strongly reduced the material which was retained by phenylboronate; (2) the ADPR transferase inhibitor benzamide (100 μM) suppressed AO-induced poly(ADP-ribosylation); (3) poly(ADP-ribosylation) reduced the electrophoretic mobility of the modified histones. Several histones were constitutively poly(ADP-ribosylated) in untreated controls: 0.03% of H2A, 0.04–0.06% of H2B, and 0.04% of H3.1 carried at least one poly(ADPR) chain of undetermined length. AO transiently increased the poly(ADPR) levels of all major histones with the exception of H1. The extent of substitution 30 min after exposure to AO generated by 50 μg/mL xanthine and 5 μg/mL xanthine oxidase was 0.8% for A24 > 0.3% for H4 > 0.1% for H3.1 = 0.1% for H3.2 = 0.1% for H2B.2 > 0.09% for H2A. Within 60 min, poly(ADPR) substitution had decreased to control levels for H3 and H4 and below control levels for H2A and H2B. The addition of benzamide (100 μM) prevented AO-induced poly(ADP-ribosylation) as well as the drop in cellular NAD levels (at 30 min). MNNG was more effective than AO in inducing histone poly(ADP-ribosylation), and to judge from the significant decreases in electrophoretic mobility, the lengths of the ADPR chains were longer. In increasing order, the extents of substitution 20 min after treatment with 5 μg/mL MNNG were 1.8% for H3 > 1.2% for H1 > 0.95% for H2B > 0.36% for H4. In addition, the variant form H2B.x and unidentified low-mobility H2B- and H4-related antigens were substantially poly(ADP-ribosylated). Our results indicate that AO and MNNG induce distinct patterns of histone poly(ADP-ribosylation). Consequently, different DNA strand breaking agents are expected to affect chromatin structure and function in a characteristic fashion.

The posttranslational modification of chromosomal proteins represents a mechanism for the structural and functional modulation of chromatin. Modification by poly(ADP-ribosylation) has been postulated to play a role in DNA replication, DNA repair (Durkacz et al., 1981; Sims et al., 1982), DNA amplification (Bürkle et al., 1987), and cell differentiation (Farzaneh et al., 1982; Althaus et al., 1982). It is a metabolic consequence of clastogens because DNA containing breaks activates the biosynthetic enzyme adenosine diphosphoribose (ADPR)[1] transferase which polymerizes NAD residues to poly(ADPR) chains (Althaus et al., 1985; Althaus & Richter, 1987). The rapid turnover of poly(ADPR) chains renders this protein modification particularly suitable for regulatory purposes. Three classes of chromosomal proteins have been found to serve as poly(ADPR) acceptors in intact cells: (1) nuclear enzymes, e.g., ADPR transferase and topoisomerase I (Singh et al., 1985; Adamietz, 1987; Krupitza & Cerutti, 1989); (2) transcription regulatory proteins, e.g., the protein encoded by the c-fos protooncogene (Krupitza and Cerutti, unpublished results); (3) structural proteins, e.g., proteins associated with the nuclear matrix (Cardenas-Corona et al., 1987; Adolph & Song, 1985) and histones (Kreimeyer et al., 1984; Adamietz & Rudolph, 1984). Enzymes are usually inactivated as a consequence of poly(ADP-ribosylation) while conformational changes result from the poly(ADPR) substitution of structural proteins (Althaus et al., 1985; Althaus & Richter, 1987). For example, the poly(ADP-ribosylation) of histones appears to relax nucleosomal cores (Poirier et al., 1982).

In the present work, we have concentrated on constitutive and clastogen-induced histone poly(ADP-ribosylation) in the intact, immortalized human keratinocytes HaCaT (Boukamp et al., 1988). Active oxygen generated extracellularly by xanthine/xanthine oxidase (Kellogg & Fridovich, 1975) and the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

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[1] Abbreviations: ADPR, adenosine diphosphoribose; AO, active oxygen; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; poly(ADP-ribose), poly(adenosine diphosphoribose); SVPD, snake venom phosphodiesterase; X/XO, xanthine/xanthine oxidase; NAD, nicotinamide adenine dinucleotide, oxidized form; AUT-PAGE, acid-urea-Triton-polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

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(MNNG) were used as clastogens. A combination of affinity chromatography on phenylboronate resin (Adamietz & Rudolph, 1984) with immunoblotting allowed the quantitation of poly(ADPR) substitution of individual histones.

Materials and Methods

Materials

Xanthine, benzamide, alcohol dehydroxygenase, and histone standard (calf thymus) were purchased from Sigma, xanthine oxidase and snake venom phosphodiesterase were from Böehringer Mannheim, phenylboronate–agarose matrix gel PBA 30, ultrafiltration cells, and YM-5 filters were from Amicon, nitrocellulose (0.2 μm) was from Cellpore and Schleicher & Schuell, and [^{3}H]leucine and ^{125}I-labeled donkey anti-rabbit Ig were from Amersham.

Human keratinocytes HaCaT (spontaneously immortalized) were received from Dr. N.E. Fusenig (DKFZ-Heidelberg, see Boukamp et al., 1988). Histone-H1 (from mouse liver) polyclonal rabbit antibodies 461b were a gift from Dr. B.D. Stollar (Tufts University, Boston, see Neary & Stollar, 1987) and Histone H2A, H2B, H3 and H4 (from calf thymus) polyclonal rabbit antibodies were a gift from Dr. M. Bustin (NCI, Bethesda, see Bustin, 1978). Mouse monoclonal antibodies against poly(ADPR) were a gift of Dr. A. Belcredi (OF2S-Austria, see Belcredi, 1987).

Methods

Cell Culture. Monolayer cultures of human keratinocytes HaCaT were grown in 15-cm-diameter petri dishes in DMEM medium supplemented with 10% fetal calf serum (FCS) and antibiotics in a 5% CO_2 atmosphere at 37 °C in a humidified incubator (Boukamp et al., 1988). Cells were grown to 70% confluency, pooled, replated at 5×10^5 cells per dish, and allowed to grow to 60% confluency in DMEM supplemented with 10% FCS and [^{3}H]leucine (1 μCi/mL). The medium was then replaced with nonradioactive M199 medium containing 10% FCS, and after 24 h, cells were exposed to active oxygen generated by xanthine (50 μg/mL) and xanthine oxidase (5 μg/mL) or to MNNG (5 μg/mL) in the presence or absence of benzamide (100 μM).

NAD Determination. Monolayer cultures were allowed to grow to 70% confluency in 15-cm-diameter petri dishes. After exposure of the cells to AO or MNNG for 20, 30, and 60 min in the presence or absence of benzamide (100 μM), the medium was removed, and the cells were washed twice with 10 mL of ice-cold PBS and lysed with 3 mL of ice-cold 0.1 N NaOH–1 mM nicotinamide. After neutralization with 0.6 mL of ice-cold 0.37 M H_3PO_4, the samples were centrifuged for 10 min at 5500*g*. The clear solution from the interphase was taken for the determination of NAD according to Jacobson and Jacobson (1979).

Separation of Histone Proteins. Cells were exposed to AO (generated by 50 μg/mL X and 5 μg/mL XO) for 30 and 60 min or to 5 μg/mL MNNG for 20 min. When indicated, the ADPR transferase inhibitor benzamide (100 μM) was added 15 min prior to treatment with the clastogen. The medium was then discarded, and the cells were lysed in ice-cold pH 3.1 buffer containing 25 mM sodium citrate, 0.5% NP-40, 2 mM benzamide, and 0.5 mM PMSF for 1 h at 4 °C, essentially as described previously (Adamietz & Rudolph, 1984). The lysed cells were scraped with a rubber policeman, and the contents of 16 plates were pooled for each experimental condition. All manipulations were at 4 °C. Nuclei were released in a glass homogenizer, layered on top of a sucrose cushion, and pelleted at 800*g* in a swing-out rotor for 10 min. Histones were extracted twice with 10 mL of 0.25 M H_2SO_4 for 30 min and once with 10 mL of 0.15 M H_2SO_4 for 15 min from the nuclear pellet (0 °C).

The acid-soluble proteins were precipitated by the addition of solid TCA to 25% final concentration and centrifuged at 5500*g* for 2 h (0 °C) (Duhl & Hnilica, 1986). The supernatant was discarded and the remaining pellet washed carefully with ethanol (−20 °C) and acetone (−20 °C). The pellets were dissolved in 8 mL of pH 6.5 buffer containing 6 M guanidinium chloride, 50 mM sodium phosphate, and 5 mM β-mercaptoethanol (Adamietz & Rudolph, 1984).

Separation of Poly(ADP-ribosylated) Proteins. The pH of the dissolved pellets containing the histones was adjusted to 8.2 by addition of 400 μL of 1 M morpholine buffer, pH 8.7, and poly(ADP-ribosylated) proteins were separated by adsorption to columns containing 2 mL of PBA30 matrix gel. Exactly the same amounts of protein according to [^{3}H]Leu radioactivity were applied onto the columns for each experimental condition. The columns were washed with 30 mL of a pH 8.2 buffer (6 M guanidinium chloride–50 mM morpholine) and poly(ADP-ribosylated) proteins eluted with 20 mL of 6 M guanidinium chloride–200 mM phosphate, pH 5.5. Eluted adsorbed material as well as nonadsorbed column flow-through and washes were pooled separately, concentrated, and dialyzed by ultrafiltration on YM-5 filters. The material retained on the filters was dissolved in AUT–PAGE sample buffer.

Separation of Histones by Acetic Acid–Urea–Triton–PAGE (AUT–PAGE) and Immunoblot Analysis with Polyclonal Antibodies. Aliquots of the phenylboronate nonadsorbed flow-through and the eluted, adsorbed material were applied to 15% AUT–PAGE (Zweidler, 1978). For each experimental condition, aliquots of the two fractions were chosen which gave the same ratio of [^{3}H]Leu radioactivity. Developed gels were equilibrated for 45 min in a pH 6.8 buffer containing 2.3% SDS–8% glycerol–0.5% β-mercaptoethanol–60 mM Tris base (Johnson et al., 1983) and proteins electrotransferred for 3 h (0.3 A, 15 V, 4 °C) to nitrocellulose (Towbin et al., 1979).

All immunoreactions were carried out in sealed plastic bags. The nitrocellulose filters were saturated overnight with 10% skimmed milk–0.1% NaN_3 at 4 °C, and specific histone antibodies (1:1000 dilution) were added. Following incubation for 12 h at 4 °C (with agitation), the filters were washed exhaustively with distilled water and finally with 10% skimmed milk. The membranes were then incubated with ^{125}I-labeled donkey anti-rabbit Ig (10^5 cpm/mL in 10% skimmed milk at 4 °C, overnight with agitation) and subsequently exposed to a presensitized Amersham MP X-ray film at −70 °C without the use of enhancers. The intensities of the bands of the autoradiograms were measured by densitometry. The ^{3}H activity of the metabolically labeled proteins did not interfere with the image of the ^{125}I-reacted immunoblot.

Immunoblotting with Anti-Poly(ADPR) Monoclonal Antibodies. After overnight saturation at 4 °C of nonspecific binding sites of nitrocellulose filters with a pH 7.4 buffer consisting of 5% FCS, 10 mM Tris, 1 × Denhardt's solution, and 0.1% NaN_3, monoclonal antibodies against poly(ADPR) chains were added at a 1:5 dilution in the above buffer and incubated for 12 h with agitation at 4 °C. The membranes were washed with Tris-buffered saline and incubated overnight with ^{125}I-labeled sheep anti-rabbit Ig (10^5 cpm/mL in above buffer) with agitation at 4 °C.

Quantitation of Histones by Immunoblotting. Increasing protein amounts from the nonabsorbed control fraction (applied on gels according to the cpm of the metabolic [^{3}H]Leu label) were separated on AUT–PAGE and blotted against

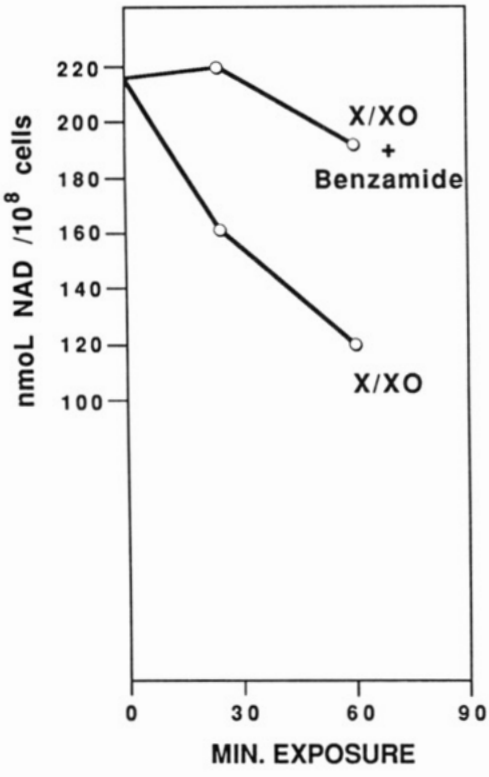

FIGURE 1: Effect of AO treatment on NAD concentrations in HaCaT cells. Monolayer cultures were treated with X/XO (50/5 μg/mL) in the presence or absence of benzamide (100 μM) and the cellular NAD concentrations determined according to Jacobson and Jacobson (1979).

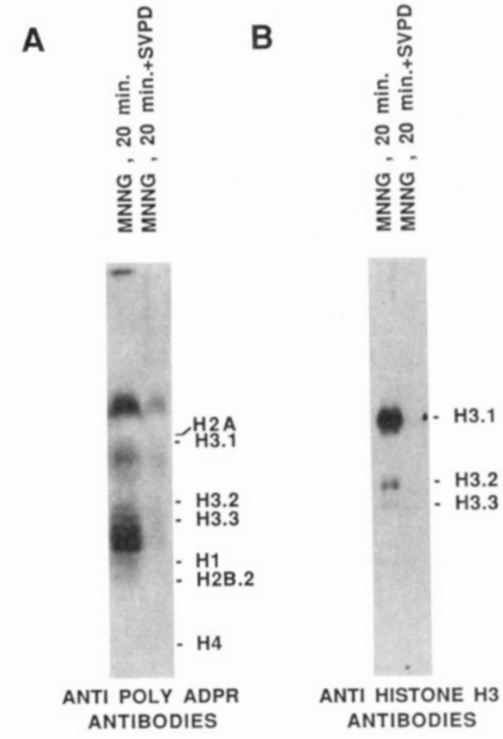

FIGURE 2: Removal of poly(ADPR) chains from acid-soluble nuclear acceptor proteins by snake venom phosphodiesterase. Acid extracts of nuclear preparations from MNNG (5 μg/mL)-treated HaCaT cells were directly purified on phenylboronate PBA30 columns or first incubated with snake venom phosphodiesterase before application to the column. The material which remained adsorbed to the column was eluted and analyzed by immunoblotting with monoclonal mouse antibody against poly(ADPR) chains (part A) or polyclonal rabbit antibody against histone H3 (part B) as described under Methods.

histone antibodies as described above, and the autofluorogram was subjected to densitometry. The antibody reactions and the darkening of the X-ray film were proportional to the applied amounts of protein.

Release of Poly(ADPR) Chains with Snake Venom Phosphodiesterase (SVPD). In order to characterize and quantitate the material retained by phenylboronate PBA30 columns, acid extracts of nuclear preparations were subjected to snake venom phosphodiesterase (SVPD) which is known to degrade poly(ADPR) chains (Chambon et al., 1966; Nishizuka et al., 1967).

For comparison, acid-soluble nuclear proteins from MNNG-treated cells were divided into two aliquots only one of which was treated with SVPD in 500 μL of 40 mM Tris (pH 8.9)–1 mM PMSF–0.25 mg of aprotinin for 3 h at 37 °C. The reaction was terminated by the addition of 9.5 mL of pH 6.5 buffer containing 6 M guanidinium chloride–50 mM sodium phosphate–5 mM β-mercaptoethanol. The pH of the solution was adjusted to 8.2 with 1 M morpholine, pH 8.7, before the samples were applied on top of phenylboronate PBA30 columns as described above. The effect of the SVPD treatment was monitored by immunoblotting with monoclonal antibodies against poly(ADPR) (Figure 2A) and polyclonal antibodies against histone H3 (Figure 2B). SVPD treatment strongly reduced the amount of total poly(ADP-ribosylated) nuclear proteins and the amounts of H3.1 and H3.2 retained by the PBA30 column. Some residual retention of H3 by the column is probably due to incomplete removal of poly(ADPR) chains rather than nonspecific adsorption of H3. Similar observations were made by other investigators (G. Sauermann, personal communication).

Results

We have investigated the poly(ADP-ribosylation) of the major histones in the immortalized human keratinocytes HaCaT following exposure to an extracellular burst of active oxygen (AO) generated by xanthine/xanthine oxidase (50 μg/mL X and 5 μg/mL XO) or the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, 5 μg/mL), respectively. The nomenclature of Zweidler (1978) for the variant proteins within the major histone groups was used. Both agents efficiently introduce DNA strand breakage, although by different mechanisms. The duration of the exposure to the strand breaking agent was 30 min for AO and 20 min for MNNG, at which time the extent of poly(ADP-ribosylation) was close to maximal (Muehlematter et al., 1988; Singh et al., 1985).

The effect of AO exposure of HaCaT cells on the cellular NAD pool is shown in Figure 1. A burst of AO generated by 50 μg/mL X and 5 μg/mL XO resulted in a 25% decrease in NAD within 30 min and a 45% decrease within 60 min. The fact that the ADPR transferase inhibitor benzamide (100 μM) effectively attenuated NAD depletion after 30 and 60 min suggests that the reaction is mostly due to NAD consumption by poly(ADPR) synthesis. Similar results were obtained upon treatment with 5 μg/mL MNNG (data not shown).

In our experimental approach for the quantitation of histone poly(ADP-ribosylation) in intact HaCaT cells, we prelabeled cellular proteins with [^{3}H]Leu before AO or MNNG treatment in order to allow accurate aliquoting during the multistep analytical procedure. Poly(ADP-ribosylated) histones were purified by affinity chromatography of acid nuclear extracts on phenylboronate PBA30 columns and identified by immunoblotting with polyclonal antibodies against the five major classes of histones. Poly(ADPR) substitution was charac-

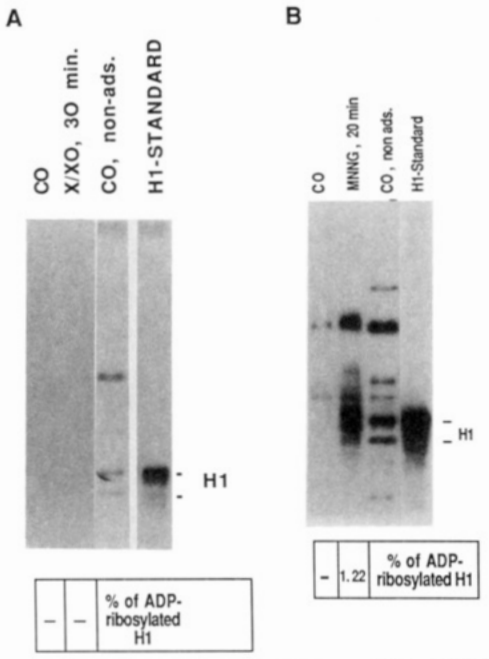

FIGURE 3: Poly(ADP-ribosylation) of histone H1 in HaCaT cells. [^{3}H]Leu-labeled HaCaT cells were treated with AO or MNNG, respectively. Acid nuclear extracts were separated by phenylboronate PBA30 affinity chromatography and the nonadsorbed flow-through and eluted adsorbed material analyzed by immunoblotting with polyclonal rabbit antibody against histone H1. The percentage of H1 molecules which carry at least one ADPR residue is indicated on the bottom and was calculated from the amounts of protein contained in the PBA30 nonadsorbed and adsorbed fractions and the densitometer readings of the immunoblots (see Methods). It represents a mean of three independent experiments which varied by ±15%. (Part A) Treatment with AO (X/XO at 50/5 μg/mL) for 30 min. (Part B) Treatment with MNNG (5 μg/mL) for 20 min.

terized by using the following criteria: (1) retention of modified protein by phenylboronate PBA30 resin; (2) removal of poly(ADPR) chains, resulting in loss of retention on PBA30 resin by treatment of nuclear extracts with snake venom phosphodiesterase; (3) suppression of poly(ADP-ribosylation) by the ADPR transferase inhibitor benzamide (100 μM). [^{3}H]Leu radioactivity measurements together with the intensities of specific bands on the autoradiograms of the immunoblots allowed the calculation of the extent of poly(ADPR) substitution of specific histones. This procedure is justified since a linear relationship was observed between the amounts of protein applied on the gels and the intensities of the bands on immunoblots. While the accuracy of the values for the extent of poly(ADPR) substitution was high for a single experiment, there was significant variation between experiments. Mean values of three experiments are listed below the immunoblots in the figures, and estimated errors are given in the legends. It should be noted that with our approach the fraction of a specific protein which carriers at least one poly(ADPR)-substituent of undetermined length is being measured.

Active Oxygen Induces Poly(ADP-ribosylation) of Core Histones. The immunoblots reproduced in Figure 3A indicate that H1 was not detectably poly(ADP-ribosylated) in response to AO produced extracellularly by xanthine/xanthine oxidase (50/5 μg/mL), in contrast to MNNG (5 μg/mL) where it serves as a major acceptor (Figure 3B, see below).

Induction of poly(ADP-ribosylation) of H2A and its ubiquitinated form A24 by exposure to AO is shown in Figure 4A which reproduces immunoblots and lists the percent of protein molecules which are substituted with at least one poly(ADPR) chain of undetermined length. It is evident that

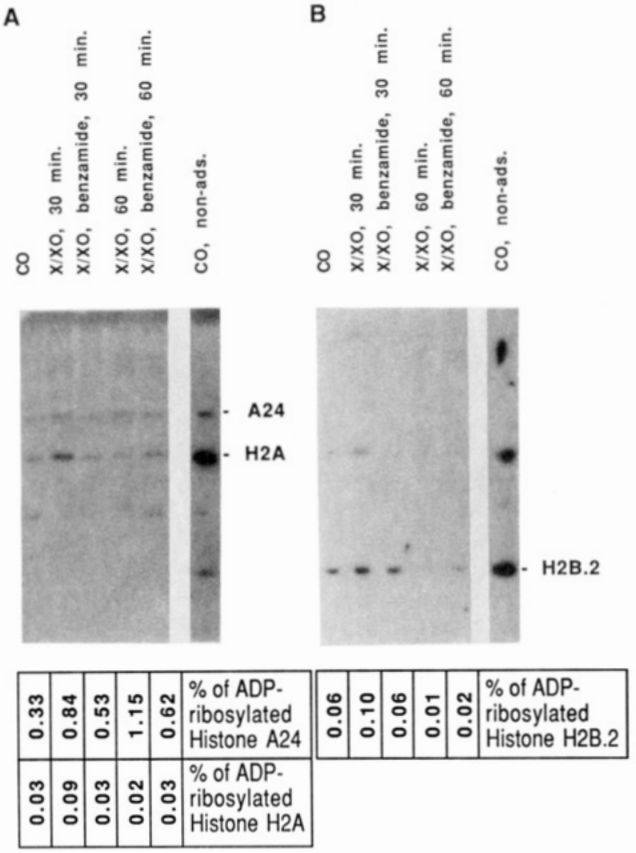

FIGURE 4: Poly(ADP-ribosylation) of histones H2A, A24, and H2B.2 by AO in HaCaT cells. [^{3}H]Leu-labeled HaCaT cells treated with AO (X/XO 50/5 μg/mL) for 30 or 60 min in the presence or absence of benzamide (100 μM). Preparation of acid nuclear extracts, affinity chromatography on phenylboronate PBA30 resin, immunoblotting, and data calculation were as described in Figure 3 and under Methods. The values for the levels of poly(ADPR) substitution are means of three independent experiments. For A24 and H2A (part A), the individual values varied by ±10% and for H2B.2 by ±18% (part B). (Part A) Immunoblots with polyclonal rabbit antibody against histone H2A. (Part B) Immunoblots with polyclonal rabbit antibody against histone H2B.

A24 and to a lower extent H2A were constitutively poly(ADP-ribosylated) and that AO induced a transient 3-fold increase in poly(ADPR) levels (lanes 1, 2, and 4). This increase was partially prevented by benzamide (lanes 3 and 5). As expected, poly(ADP-ribosylation) slightly reduced the mobilities of H2A and A24. The maximal extent of substitution of A24 was 13 times higher than that of H2A. Analogous results for H2B.2 are shown in Figure 4B. The data indicate that H2B.2 represents a relatively poor poly(ADPR) acceptor following AO treatment, the increase in the poly(ADPR) level after 30 min being only 2-fold. The identity of the low-mobility band on the immunoblots is not known. It could represent ubiquitinated H2B or a protein which cross-reacts with our antibody.

Immunoblots with polyclonal antibodies against histone H3 are shown on Figure 5A. From lane 6, which shows the immunoblot of the non-poly(ADP-ribosylated), acid-soluble nuclear proteins contained in the flow-through of the phenylboronate column (lane 6), it is evident that our antibody recognizes H3.1, H3.2, and H3.3 of the histone H3 group. Lane 1, which contains affinity chromatography purified material of an untreated control, shows that only H3.1 is constitutively poly(ADP-ribosylated). AO treatment substantially increased the poly(ADP-ribosylation) of H3.1 and H3.2 to a level where 0.11% of each protein molecule on average carried at least one poly(ADPR) substituent. The

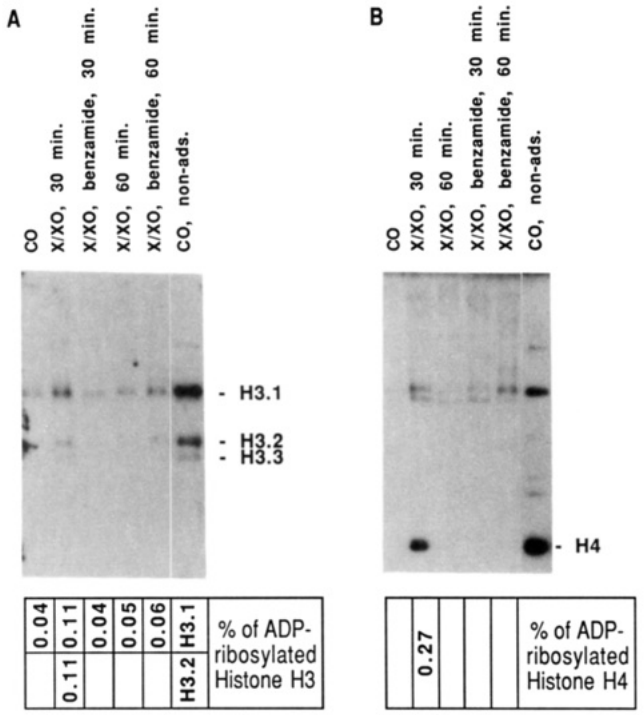

FIGURE 5: Poly(ADP-ribosylation) of histones H3 and H4 by AO in HaCaT cells. [^{3}H]Leu-labeled HaCaT cells were treated with AO (X/XO 50/5 μg/mL) for 30 or 60 min in the presence or absence of benzamide (100 μM). Preparation of acid nuclear extracts, affinity chromatography on phenylboronate PBA30 resin, immunoblotting, and data calculation were as described in Figure 4 and under Methods. The values for the levels of poly(ADPR) substitution are means of three independent experiments. For H3, the individual values varied by ±25% (part A) and for H4 by ±15% (part B). (Part A) Immunoblots with polyclonal rabbit antibody against histone H3. (Part B) Immunoblots with polyclonal rabbit antibody against histone H4.

reaction was completely suppressed in the presence of the ADPR transferase inhibitor benzamide. Figure 5B contains the corresponding results with polyclonal antibody against histone H4. While H4 is not detectably poly(ADP-ribosylated) in control cells (lane 1), it serves as a strong poly(ADPR) acceptor with a level of substitution of 0.27% 30 min after exposure to AO (lane 2).

In summary, all core histones but not H1 served as poly(ADPR) acceptors after exposure to AO, but the extent of substitution varied substantially. The extents of substitution in decreasing order were A24 > H4 > H3.1 = H3.2 = H2B.2 > H2A. Substantial constitutive poly(ADPR) substitution was found on A24, H3, and H2A.

N-Methyl-N'-nitro-N-nitrosoguanidine-Induced Histone Poly(ADP-ribosylation). The immunoblots reproduced in Figure 3B indicate that, in contrast to AO, MNNG (5 μg/mL) strongly induced histone H1 poly(ADP-ribosylation) to 1.2% substitution within 20 min. The fact that modified H1 and its variants possessed decreased electrophoretic mobilities relative to the phenylboronate flow-through and the authentic standard indicates that the protein is substituted with oligo- and poly(ADPR) chains. There is no evidence for constitutive poly(ADP-ribosylation) of H1 from the immunoblot of the untreated control (lane 1).

The poly(ADP-ribosylation) of the core histones in response to MNNG treatment is shown in Figure 6. It is noteworthy that the immunoblots of phenylboronate flow-through material and authentic standards with antibodies raised against H2B and H4 contain unidentified low-mobility bands (Figure 6B, lanes 3 and 4; Figure 6D, lanes 3 and 4). They may consist of cross-reacting related antigens or ubiquitinated derivatives of the parent histones. Low levels of constitutive poly(ADP-ribosylation) are detectable in the PBA30-adsorbed materials of untreated controls for H2A (0.03%), H2B2.2 (0.04%), and H3.1 (0.04%) and the low-mobility band of H4. Significant shifts to lower electrophoretic mobilities are observed for each modified protein, indicating that they are substituted by oligo- or poly(ADPR) chains. The extents of substitution with at least one poly(ADPR) chain of undetermined length are, in decreasing order, 1.8% for the H3 group, 0.95% for total H2B (disregarding the low-mobility protein), 0.36% for H4 (disregarding the low-mobility protein), and 0.32% for H2A. The variant form H2B.x was only present in very small amounts in untreated HaCaT cells but became visible in immunoblots of the nonadsorbed PBA30 flow-through when larger aliquots were applied on the gel (not shown). However, H2B.x is very efficiently poly(ADP-ribosylated) in response to MNNG since a band was readily discernible in the immunoblot of the PBA30-adsorbed material (Figure 6B, lane 2). A substitution level of 5.8% was calculated for H2B.x.

Discussion

Our work indicates that an extracellular burst of active oxygen (AO) induces the poly(ADP-ribosylation) of all core histones in human keratinocytes HaCaT while "the linker" histone H1 was not detectably substituted. The different classes of core histones were unevenly modified. In decreasing order, the degree of substitution following AO exposure for 30 min was A24 > H4 > H3 > H2B > 2A. In part, these differences in the extent of substitution of individual histones are probably determined by DNA–protein and protein–protein interactions in nucleosomes and higher order chromatin structures which may affect accessibility of ADPR transferase and glycohydrolase (Althaus et al., 1985; Althaus & Richter, 1987). It follows from our results that different DNA breaking agents induce different patterns of histone poly(ADP-ribosylation). In contrast to AO, the methylating agent MNNG induced preferential poly(ADP-ribosylation) of H3, H1, and H2B. Preferential poly(ADP-ribosylation) of H2B, A24, and H3 had been reported in phorbol 12-myristate-13-acetate-treated mouse embryo fibroblasts (Singh & Cerutti, 1985) and of H2B in dimethyl sulfate treated rat hepatoma cells (Adamietz & Rudolph, 1984). H2B and H3 were also highly accessible to poly(ADP-ribosylation) in isolated nuclei from dimethyl sulfate treated cells (Boulikas, 1988). The chemistry and repairability of DNA breaks induced by different clastogens may affect the pattern of poly(ADPR) substitution. The majority of breaks by AO are induced by a Ca^{2+}-requiring process which may involve the activation of a Ca^{2+}-dependent endonuclease (Muehlematter et al., 1988) while MNNG mostly causes breakage at apurinic sites in G-rich regions. DNA breaks by singlet oxygen have been reported to occur preferentially at kinks 1.5 helical turns from the center of nucleosomal DNA (Hogan et al., 1987).

Only slight or no decreases in the electrophoretic mobilities of the histones upon ADP-ribosylation were observed in our experiments with AO. Therefore, it is likely that the lengths of the ADPR chains are short. As discussed in the literature, nuclear mono- and oligo(ADP-ribosyl) substituents from intact cells are probably relatively stable remnants of longer chains which have been shortened by the action of glycohydrolase. Direct nuclear mono(ADP-ribosylation) appears unlikely (Althaus et al., 1985; Althaus & Richter, 1987). Adamietz and Rudolph (1984) estimated that mono(ADPR) substituents were at least 2 times more abundant than poly(ADPR) chains in acid-extractable nuclear proteins from dimethyl sulfate treated rat hepatoma cells. In contrast to AO, poly(ADP-

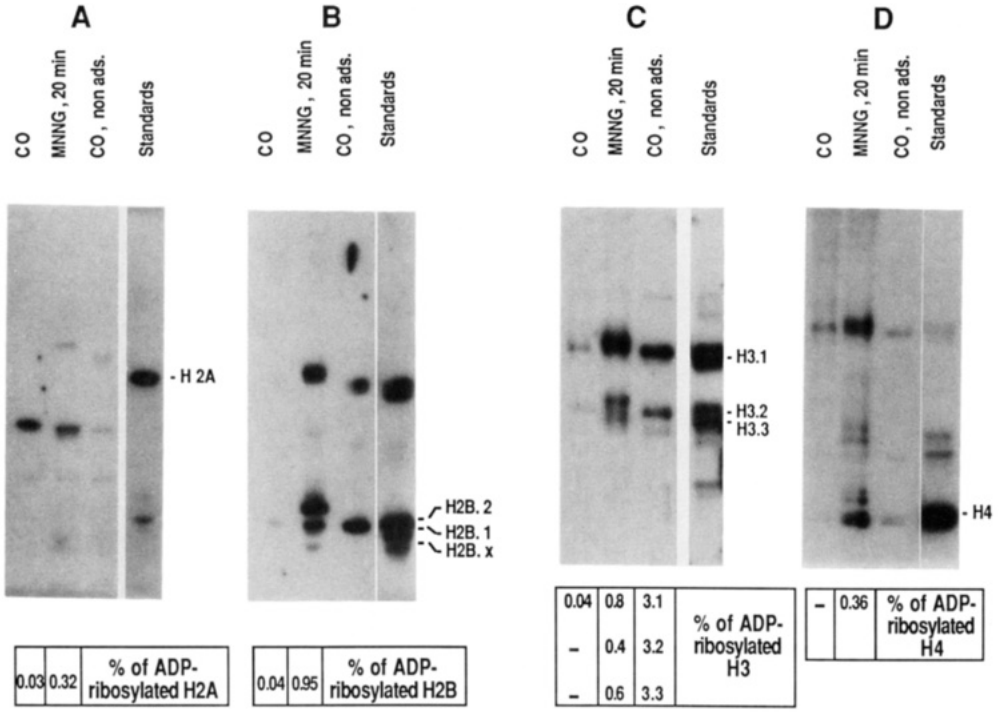

FIGURE 6: Poly(ADP-ribosylation) of core histones by MNNG in HaCaT cells. [^{3}H]Leu-labeled HaCaT cells were treated for 20 min with 5 μg/mL MNNG. Preparation of acid nuclear extracts, affinity chromatography on phenylboronate PBA30 resin, immunoblotting, and data calculation were as described in Figure 3 and under Methods. The values for the levels of poly(ADPR) substitution are means of three independent experiments which varied by ±5–10%. (Part A) Immunoblots with polyclonal antibody against histone H2A. (Part B) Immunoblots with polyclonal antibody against histone H2B. (Part C) Immunoblots with polyclonal antibody against histone H3. (Part D) Immunoblots with polyclonal antibody against histone H4.

ribosylation) of the histones by MNNG caused substantial decreases in their electrophoretic mobilities, indicating longer average lengths of the poly(ADPR) chains. The reasons for this difference between AO- and MNNG-induced poly(ADP-ribosylation) are not known. Poly(ADPR) chains with different branching structures may be synthesized in response to the two clastogens, resulting in different susceptibilities to degradation by glycohydrolase.

A24 was by far the most highly ADP-ribosylated acid-soluble nuclear protein in AO-treated HaCaT cells. A24, which is the ubiquitinated form of histone H2A, was 13 times more extensively modified than the parent protein. It is unknown whether poly(ADPR) substitution takes place at the ubiquitin or H2A portion of the molecule. A low-mobility band of ADP-ribosylated material was also observed on the immunoblot with H2B-specific antibody and might consist of ubiquitinated H2B. The preferential ADP-ribosylation of A24 and possibly of ubiquitinated H2B is intriguing in the context of the recent finding that the radiosensitivity gene RAD6 of yeast encodes an enzyme which accomplishes the ubiquitination of H2B. It was speculated that ubiquitination plays a role in "chromatin remodelling" which may be required to mark chromatin regions for active transcription and repair (Jentsch et al., 1987). Preferential ADP-ribosylation of ubiquitinated histones might further contribute to the local relaxation of nucleosomal and higher order chromatin structures. It is interesting to note that another protein of the H2B group, H2B.x, was by far the most extensively poly(ADP-ribosylated) histone following MNNG treatment.

According to present models of nucleosomes, histones H4 and H3 are anchored more tightly in the core structure than H2A and H2B (Jackson & Chalkey, 1981; Louters & Chalkey, 1985). It has been reported that poly(ADP-ribosylation) of H3 and H4 results in their release from DNA (Wesierska-Gadek & Sauermann, 1988). AO efficiently induced H4 ADP-ribosylation in HaCaT cells. Therefore, it is likely AO has profound effects on nucleosomal structure in the neighborhood of DNA breaks. Nucleosomal displacement may be required to allow the transcription of certain genes. For example, a nucleosome-free region extending from base pairs −200 to +500 has been discovered at the mouse β-major globin locus in Friend cells in which the gene can be transcriptionally activated (Benezra et al., 1986). Loss or alteration of a single nucleosome in the promoter region accompanies the induction of mouse mammary virus sequences by steroid hormone (Richard-Foy & Hager, 1987).

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Registry No. MNNG, 70-25-7; ADPR transferase, 58319-92-9.

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Cultures of Separated Mating Types of *Blakeslea trispora* Make D and E Forms of Trisporic Acids†

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ABSTRACT: Trisporic acids are end products of the sex-specific pheromones in mucoraceous fungi. We have found three new trisporic acids in cultures of *Blakeslea trispora* in which (+) and (−) mating types were separated by a membrane with 0.45-μm pores. Two of the trisporic acids were new compounds; the structure of the third [previously described by Miller and Sutter [(1984) *J. Biol. Chem. 259*, 6420] as methyl trisporate-E with a hydroxyl group at C-2] was revised. Trisporic acid-E(3*R*), trisporic acid-E(3*S*), and trisporic acid-D(2*S*) were in a 1:1:2 ratio, accounted for 9% of the total trisporic acids, and differed by the position and configuration of a hydroxyl group on the ring at C-2 or C-3, the conformation of the ring, the extent of rotation of the side chain relative to the ring, and either a carbonyl or hydroxyl group on the side chain at C-13. These three compounds accounted for only 0.5% of the total trisporic acids in combined mating type cultures. Since the combined cultures did not metabolize trisporic acid-E(3*R*), its biosynthesis apparently ceases when opposing mating types contact each other physically. We speculate that *B. trispora* and *Phycomyces blakesleeanus* utilize different pheromones to regulate an early event (possibly zygotropism) in sexual development.

Trisporic acids are oxygenated, 18 carbon atom derivatives of β-carotene (Figure 1; Caglioti et al., 1966). Trisporic acids normally are found in the medium of combined, but not separate, (+) and (−) mating type cultures of mucoraceous fungi (van den Ende, 1978; Jones et al., 1981; Sutter, 1987). Trisporic acids stimulate carotenogenesis in *Blakeslea trispora* and the development of zygophores (sexual hyphae) in *Mucor mucedo* (Caglioti et al., 1966; van den Ende, 1968). However, physiological concentrations of (extracellular) trisporic acids stimulate neither carotenogenesis nor the development of zygophores in *Phycomyces blakesleeanus* nor the development

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