Increased nuclear conjugated polyamines and transglutaminase during liver regeneration

(putrescine/spermidine/spermine/proliferation)

MARI K. HADDOX AND DIANE HADDOCK RUSSELL

Department of Pharmacology, University of Arizona Health Sciences Center, Tucson, Arizona 85724

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ABSTRACT The nuclear content of conjugated polyamines increased during rat liver regeneration. Conjugated polyamines isolated from the acid-precipitable fraction of nuclei required peptide bond hydrolysis for release of the parent compounds. The most striking change occurred in conjugated putrescine which fluctuated in a biphasic manner; maximal nuclear levels 12-fold and 25-fold above those of sham-operated controls were achieved at 4 and 42 hr after hepatectomy, respectively. Conjugated spermidine and spermine increased 3- and 2-fold respectively within 4 hr and remained high throughout the 48 hr studied. When expressed on the basis of mg of nuclear protein, the maximal conjugated putrescine increased 19-fold, conjugated spermidine increased 2-fold, and conjugated spermine decreased by 50%. Therefore, the spermidine and spermine conjugates may be of a more constitutive nature whereas the large changes in the nuclear conjugation of putrescine associated with the onset of growth may play a regulatory role. The nucleus also contained transglutaminase (R-glutaminyl-peptide:amine y-glutamyl-ytransferase, EC 2.3.2.13), an enzyme shown in vitro to conjugate polyamines covalently to proteins. The specific activity of the nuclear enzyme increased rapidly after partial hepatectomy to a level 3-fold above control at 4 hr and 7-fold above control at 42 hr. The increased conjugating activity resulted from an increase in detectable maximal velocity and not a change in affinity of the enzyme for putrescine (Km = 0.4 mM). There was also a 3-fold increase at 42 hr in the number of nuclear amine acceptor sites present to which radiolabeled putrescine could be conjugated by endogenous enzyme.

The diamine putrescine and the polyamines spermidine and spermine are small aliphatic nitrogenous bases which function as the organic cations of the cell and have known effects on macromolecular biosynthesis (1–4). Increased polyamine biosynthesis and accumulation occur in all growth responses studied to date, including embryonic development, tissue regeneration, cellular hypertrophy, and cell cycle traverse in culture (3, 4). Because the amino groups of these compounds are fully charged at physiological pH, the polyamines can interact with negatively charged cellular constituents such as nucleic acids and proteins. This electrostatic binding is believed to be the basis of several of the known effects that polyamines have on nucleic acid conformation and biosynthetic enzyme activity (1, 2).

We have recently demonstrated (5) the existence of another mechanism of action by which polyamines could modify mammalian cell function: the posttranslational conjugation of polyamines to proteins. Covalent conjugates of putrescine, spermidine, and spermine, which required peptide bond hydrolysis for release of the parent compound, were isolated from liver nuclear acid precipitates. Furthermore, the nucleus also contains a putative conjugating enzyme, transglutaminase (R-glutaminyl-peptide:amine y-glutamyl-ytransferase, EC 2.3.2.13), as well as amine acceptor protein substrates to which radiolabeled polyamines can be conjugated by endogenous enzyme (5).

Polymamines exist in bacteria in several conjugated forms. In stationary-phase Escherichia coli cells, spermidine is conjugated to glutathione; the physiological significance of this is unknown (6–8). Bacillus brevis strain Vm4 conjugates spermidine to a pentapeptide to form the antibiotic edeine (9, 10) which inhibits DNA (11, 12) and protein (13) synthesis. The cyanobacterium Anacystis nidulans, which concentrates extracellular putrescine to lethal intracellular concentrations when grown in an alkaline environment (14), contains ca. 0.2% of the accumulated diamine in an acid-insoluble protein fraction (15). At least some of this putrescine is covalently linked to the 30S and 50S ribosomal subunits, and an increase in the amount of this conjugate is associated with irreversible dissociation and thereby inactivation of the 70S ribosome (15).

To determine if conjugated polyamines in mammalian cells might have regulatory roles, we determined whether the amount of nuclear polyamine conjugates was altered as a function of growth state. We found that a rapid increase in the amount of conjugated polyamines and transglutaminase activity occurred in rat liver nuclei in response to partial hepatectomy.

MATERIALS AND METHODS

Partial hepatectomy (16) was performed on ether-anesthetized Sprague–Dawley male rats (120–150 g). Sham-operated animals were anesthetized and laparotomized. Livers were homogenized in 5 vol of 2.1 M sucrose/9 mM MgCl2/0.1 mM spermine (17) by a Tekmar Tissumizer (18) at maximum speed for three 1-min periods with intermittent 1-min rests. The homogenate was filtered through 10 layers of cheesecloth and the filtrate was spun at 32,000 × g for 90 min. The crude nuclear pellets were washed twice by suspension in 0.5 vol of 0.88 M sucrose/0.05 mM MgCl2/0.2% Triton X-100 and centrifugation at 1900 × g for 15 min (19, 20) and were finally suspended (50–100 × 106/ml) in 0.34 M sucrose/0.05 mM MgCl2/1 mM phenylmethylsulfonyl fluoride and stored in liquid nitrogen. Isolated nuclei were counted by using a hemocytometer after dilution (1:10) in 0.3 M sucrose/0.2% Triton X-100/0.5% toluidine blue. Protein content was assessed by the Bradford dye binding method (21).

Transglutaminase activity was assayed in triplicate as the Ca2+-dependent covalent incorporation of [14C]putrescine into acid-precipitable protein (22). The assay reaction mixture (75 μl) contained 10 mM Tris-HCl at pH 7.5, 30 mM NaCl, 10 mM dithiothreitol, 5 mM CaCl2, dimethylcasein at 5 mg/ml, and the indicated concentrations of putrescine containing 1.0 μCi (75...
of $^{[14]C}$putrescine (102 mCi/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels; New England Nuclear). The reaction was initiated by the addition of 10 µl of nuclei; the mixture was incubated at 37°C, and 50-µl aliquots were spotted on filter papers (Whatman 3MM) and precipitated by addition to 10% trichloroacetic acid. The papers were washed twice more with acid, dried, and assayed in a toluene-based scintillant. The conjugation of $^{[14]C}$putrescine was linear with time and the amount of nuclei assayed and was totally dependent on the presence of Ca$^{2+}$.

The polyamine content of isolated nuclear preparations was determined by high-pressure ion-exchange chromatography utilizing a Durrum D-500 automatic amino acid analyzer as described (23, 24). Sedimented nuclei were extracted with 5 vol of 10% trichloroacetic acid, the supernatant was analyzed directly for free polyamine content, and the precipitate was washed with acid (0.1 M HCl, two times), ether (3 vol, three times), and butanol (after solubilization of the pellet in 2 M NaOH and salt saturation of the suspension with a 7.1 mixture of Na$_2$SO$_4$/Na$_2$PO$_4$ at 1.2 g/5 ml) to remove electrostatically complexed polyamines before hydrolysis to release the conjugated polyamines. Analysis of precipitates extracted from nuclei that had been isolated from 200 g of liver homogenized in the presence of 200 µCi of $^{[3]H}$putrescine, 100 µCi of $^{[14]C}$spermidine, and 100 µCi of $^{[14]C}$spermine showed that no acid-soluble radioactivity remained in the precipitate after this washing procedure. The acid precipitate was finally suspended in 5 ml of 6 M HCl (containing 200 pmol of 3,3,3'-iminobispropylamine to serve as an internal standard for estimation of recovery) and incubated for 16 hr at 100°C. The polyamines were extracted from the hydrolysate with butanol (25), adjusted to 0.1 M in HCl, evaporated, and reconstituted in 200 µl of 0.1 M HCl.

RESULTS

The nuclear content of both free and conjugated polyamines increased during liver regeneration (Fig. 1). Within 4 hr of partial hepatectomy, the nuclear content of free putrescine increased 6-fold, from 4.6 to 26.6 attomoles per nucleus, and the conjugated putrescine increased 12-fold, from 0.1 to 1.2 attomoles per nucleus. Free and conjugated putrescine increased again at 36–42 hr to levels 13-fold and 25-fold greater than the respective controls. Four hours after partial hepatectomy, conjugated spermidine increased 3-fold, from 2.6 to 8.1 attomoles per nucleus, and conjugated spermine increased 2-fold, from 0.75 to 1.6 attomoles per nucleus. Maximal free nuclear polyamine levels were attained 42–48 hr after hepatectomy, at which time spermidine (1646 attomoles per nucleus) was 3.5-fold above control and spermine (751 attomoles per nucleus) was 1.7-fold above control.

Although the possibility of redistribution of the free polyamines during the nuclear isolation cannot be excluded, these levels represent nuclear concentrations [estimated by using an average rat liver nuclear volume of 200 µm$^3$ (27)] of spermidine and spermine of 4.1 and 1.5 mM, respectively, which are consistent with those obtained for cells enucleated with cytochalasin B, a technique that avoids exposure of the nucleus to nonphysiological ionic conditions (27). Furthermore, although these polyamines are termed "free" because they are not covalently bound, they are apparently present in an ionically bound form because they are not freely diffusible in the isotonic nuclear suspension buffer. Incubation of nuclei in 0.34 M sucrose for 8 hr at 4°C resulted in the loss of less than 5% of the total nuclear polyamines into the incubation medium; however, extraction (three times) of the nuclear pellet by suspension in 10 vol of 0.075 M NaCl containing 0.025 M EDTA (pH 8.0) released 78% of the total nuclear acid-soluble putrescine, 89% of the spermidine, and 81% of the spermine (unpublished data).

Peptide bond hydrolysis was necessary for the release of polyamines from the acid-precipitable fraction of rat liver nuclei (Table 1). Washed nuclear acid precipitates were divided into duplicate fractions and maintained at 4°C (nonhydrolyzed) or 100°C (hydrolyzed) for 16 hr in 6 M HCl prior to butanol extraction of the polyamines. Both fractions had similar recoveries.

Table 1. Peptide bond hydrolysis required for release of acid-precipitable nuclear polyamines

<table>
<thead>
<tr>
<th>Polyamines, attomoles/nucleus</th>
<th>Nonhydrolyzed</th>
<th>Hydrolyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>At 4 hr:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Putrescine</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0.9</td>
<td>19.5</td>
</tr>
<tr>
<td>Spermine</td>
<td>0</td>
<td>7.9</td>
</tr>
<tr>
<td>At 42 hr:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Putrescine</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1.8</td>
<td>19.7</td>
</tr>
<tr>
<td>Spermine</td>
<td>0</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Nuclei were isolated from liver 4 and 42 hr after partial hepatectomy. The precipitate obtained after extraction of the nuclei (200 x 10$^6$) with 10% trichloroacetic acid was acid- and butanol-washed, divided into two replicate fractions in 6 M HCl, and maintained for 16 hr at 4°C (nonhydrolyzed) or 100°C (hydrolyzed) prior to butanol extraction and analysis for polyamine content.
(65%) through this procedure as indicated by the similar amount of added internal standard detected. The nonhydrolyzed nuclear precipitates had no detectable butanol-extractable putrescine or spermine and only ca. 1.4 attomoles of spermidine per nucleus. In contrast, the hydrolyzed precipitates contained an average of 2 attomoles of putrescine, 19 attomoles of spermidine, and 7 attomoles of spermine per nucleus. Absolute values obtained for the conjugated polyamines varied in a 2- to 3-fold range from experiment to experiment. However, the covalently modified forms were always present and were always increased in a consistent manner after partial hepatectomy. The variation may represent differences in the recovery of the conjugates through the extensive washing procedures required prior to hydrolysis, the point at which a recovery marker can be added.

The putative polyamine conjugating enzyme transglutamininase is stimulated by Ca\(^{2+}\) (29, 30). Therefore, to minimize artifactual synthesis of polyamine conjugates during the nuclear isolation, a procedure was used that had been developed to circumvent the conventional practice of including Ca\(^{2+}\) at millimolar concentrations in the initial homogenization media (17). The requirement for cations to stabilize nuclear structure was met by including Mg\(^{2+}\) and spermine. However, because spermine, a strong base, could displace the endogenous polyamines that are weaker bases, a nuclear preparation was isolated from 42-hr regenerating rat liver in the presence and absence of spermine to determine the effect of including the polyamine in the homogenization medium. Conjugated polyamines were still detectable in the nuclei isolated in the presence of Mg\(^{2+}\) alone, although to a lesser degree; the inclusion of spermine resulted in a 2.6-fold greater detectable nuclear content of acid-insoluble putrescine and spermidine and a 6-fold increase in detectable conjugated spermine. These differences could reflect either (i) a stabilizing effect of spermine to enhance recovery of the nuclear proteins to which the polyamines are conjugated or (ii) an artifactual consequence of the addition of spermine to promote formation of the polyamine conjugates during the isolation procedure. Comparison of the specific activities of the polyamines released by acid hydrolysis of a nuclear preparation isolated in the presence of spermine and tracer amounts of radioactive putrescine, spermidine, and spermine and of the specific activities of the polyamines in the initial tissue homogenate indicated that the lesser values found in nuclei isolated without spermine were largely the result of poor recovery of endogenously formed polyamine conjugates; at most, 20% of the conjugated polyamines detectable in nuclei isolated in the presence of spermine were the consequence of the nuclear isolation and extraction procedure.

The increases in conjugated nuclear polyamines that occurred during liver regeneration were examined on the basis of nuclear protein to determine if they were occurring concomitantly with the cell doubling process. The average protein content of the nucleus isolated from control liver, 38 pg, began to increase within 4 hr after hepatectomy so that, after 16 hr, the content was 76.9 pg per nucleus and by 36 hr a maximum of 101 pg per nucleus had been achieved. When expressed per nuclear protein, the change in the nuclear conjugated putrescine was still striking, increasing from a control value of 2 pmol/mg of protein to a maximum of 2000 pmol/mg protein by 240 hr after partial hepatectomy.

**Fig. 2.** Increased nuclear transglutaminase activity during liver regeneration. Nuclei were isolated from rat liver at the times indicated after partial hepatectomy or from sham-operated controls (zero-time value) and were assayed for transglutaminase activity in the presence of 4 mM putrescine. (Inset): Double-reciprocal plot of transglutaminase velocity \((V = \text{pmol/min per mg of protein})\) vs. changing putrescine concentration \((S = \text{putrescine in mM})\) for nuclei isolated from sham-operated (○) or 42-hr regenerating (●) animals.

**Fig. 3.** Increased nuclear amine acceptor sites for putrescine conjugation during liver regeneration. Nuclei were isolated from rat liver at the times indicated after partial hepatectomy or from sham-operated controls (zero-time value) and were incubated under transglutaminase assay conditions (2 mM putrescine) in the absence of casein for 180 min at 37°C prior to determination of the amount of acid-precipitable radionuclide. (Inset) Nuclei were incubated under transglutaminase assay conditions in the absence of casein for the times indicated.
The nuclear level of transglutaminase also increased during liver regeneration. Attention has been focused on the enzyme in the past because of its role in cellular events dependent on protein crosslinking, such as blood coagulation and semen clotting (29, 30). However, the discoveries that (i) polyamines serve as substrates for the enzyme in vitro (31–33), (ii) polyamine-protein conjugates occur in vivo (5), (iii) polyamine-protein conjugates can be isolated from discrete classes of nonhistone chromosomal proteins (5), and (iv) the nuclear level of both the polyamine conjugates and transglutaminase increase concurrently during liver growth suggest that the enzyme also may be involved in the regulation of nuclear events. Direct proof that the increased nuclear transglutaminase is responsible for the nuclear conjugated polyamines requires the demonstration that the derivatized polyamines are covalently attached through an isopeptide linkage to γ-carboxamidc groups of glutamine residues in the nuclear proteins.

Previous studies on liver transglutaminase have shown that 94% of the enzyme is found in the 105,000 × g supernatant fraction and that little change in the activity of this soluble enzyme occurs during changing growth states (39). The nuclear enzyme described herein constitutes a small fraction of the total cellular enzyme (i.e., 5% compared to the activity not sedimented by the first centrifugation of the nuclear isolation); however, it increases specifically during the liver growth process whereas no change is detectable in the non-nuclear enzyme (data not shown). Studies of cultured cell lines have shown an inverse relationship between the level of cellular transglutaminase and the growth state of the cells (40). A report (41) on mitogen-stimulated lymphocytes described an early increase in transglutaminase to 6–7-fold above control within 30 min but again described a decreased level of total cellular enzyme in actively growing cells compared to control cultures. Therefore, if the increases noted here in proliferating liver nuclei also occur in other growing cells, it appears that the nuclear enzyme may be regulated independently of the cytoplasmic or membrane-associated activity.

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