(*13*). However, preliminary studies indicate that chloroamines are inferior to HOCl as activators of the latent enzyme (*14*). All previous reports have emphasized the destructive properties of HOCl (*9*). This species is used by neutrophils to destroy microbes, mammalian cells, inflammatory mediators, and various enzymes (*9*). Indeed, increased quantities of APMA-activated collagenase were recovered when neutrophils were incubated with catalase, azide, or methionine (column 2 in Table 1). Apparently HOCl can either promote or suppress collagenase activity, but in every situation examined, the oxidative activation predominated.

The reaction scheme underlying HOCl-dependent collagenase activation is unknown and is limited by our incomplete understanding of the molecular basis for the enzymes' latency. HOCl may lead to the dissociation of an enzyme-inhibitor complex, the perturbation of the molecular confirmation of a proenzyme, or the activation of a latent collagenase proactivator (*2, 4, 8*). Nonetheless, our studies with the intact neutrophil demonstrate the critical position that HOCl holds in the activation sequence. We conclude that human neutrophils are able to harness the nonspecific reactivity of HOCl by coupling its generation with the activation of an enzyme capable of mediating specific connective tissue damage. Oxidative regulation of neutrophil collagenase and possibly other tissue collagenases may represent an important step in the pathogenesis of inflammation in vivo and a rationale for the design of specific therapeutic interventions.

S. J. WEISS*
G. PEPPIN, X. ORTIZ
C. RAGSDALE, S. T. TEST
Simpson Memorial Research Institute, Division of Hematology and Oncology, Departments of Internal Medicine and *Pediatrics, University of Michigan, Ann Arbor 48109*

References and Notes

1. T. Linsenmayer, in *Cell Biology of the Extracellular Matrix*, E. Hay, Ed. (Plenum, New York, 1982), pp. 5–32; P. Bornstein and H. Sage, *Annu. Rev. Biochem.* **49**, 957 (1980).
2. J. Gross, in *Cell Biology of the Extracellular Matrix*, E. Hay, Ed. (Plenum, New York, 1982), pp. 217–253; Z. Werb, in *Collagen in Health and Disease*, J. Weiss and M. Jayson, Eds. (Churchill-Livingstone, Edinburgh, 1982), pp. 121–134; R. Perez-Tamayo, in *ibid.*, pp. 135–159.
3. P. Ward, Ed., *Handbook of Inflammation* (Elsevier, Amsterdam, 1983), vol. 4.
4. G. Lazarus, R. Brown, J. Daniels, H. Fullmer, *Science* **159**, 1483 (1968). For recent references see, H. Macartney and H. Tschesche, *Eur. J. Biochem.* **130**, 71 (1983); G. Murphy, J. Reynolds, V. Bretz, U. Baggiolini, *Biochem. J.* **203**, 209 (1982); K. Hasty, M. Hibbs, A. Kang, C. Mainardi, *J. Exp. Med.* **159**, 1455 (1984).
5. S. Weiss, R. Klein, A. Slivka, M. Wei, *J. Clin. Invest.* **70**, 598 (1982).
6. Supernatants were treated with phenylmethylsulfonyl fluoride to prevent neutrophil serine proteinases from attacking the nonhelical portions of type I collagen [A. Barrent, *Agents Actions* **8**, 11 (1978)].
7. Type I collagen was extracted from rat tails and labeled by the method of T. Cawston and G. Murphy [*Methods Enzymol.* **80**, 711 (1981)].
8. V. Uitto, H. Turto, A. Huttenen, S. Lindy, J. Uitto, *Biochim. Biophys. Acta* **613**, 168 (1980); H. Macartney and H. Tschesche, *FEBS Lett.* **119**, 327 (1980); H. Nagase, R. Jackson, C. Brinckerhoff, C. Vater, E. Harris, *J. Biol. Chem.* **256**, 11951 (1981); C. Vater, H. Nagase, E. Harris, *ibid.* **258**, 9374 (1983).
9. S. Weiss, in (*3*), pp. 37–87.
10. Latent collagenases could be autoactivated by serine proteinases (*2, 4*), but neutrophils triggered in the presence of catalase were incapable of activating the collagenase during the 15-minute incubation before phenylmethylsulfonyl fluoride was added. Increasing the incubation period to 60 minutes before the addition of phenylmethylsulfonyl fluoride did not significantly change these findings. Plasmin may also activate collagenases (*2, 4*), but neutrophils triggered in the presence of catalase and plasminogen did not activate their collagenase under these conditions (unpublished observation).
11. S. Test and S. Weiss, *J. Biol. Chem.* **259**, 399 (1984).
12. S. Weiss and S. Regiani, *J. Clin. Invest.* **73**, 1297 (1984).
13. S. Weiss, M. Lampert, S. Test, *Science* **222**, 625 (1983); S. Test *et al.*, *J. Clin. Invest.* **74**, 1341 (1984); M. Grisham, M. Jefferson, D. Melton, E. Thomas, *J. Biol. Chem.* **259**, 1040 (1984).
14. Oxidized glutathione disulfide (GSSG) has been reported to be an activator of purified neutrophil collagenase (*4, 9*). Both HOCl and *N*-chloroamines can oxidize glutathione to its disulfide, but 10^7 neutrophils can release no more than 7 nmol of GSSG if all their intracellular glutathione is oxidized and released [see A. Voetman *et al.*, *Blood* **55**, 741 (1980)]. In our system, the addition of 50 nmol of GSSG failed to activate the latent collagenase.
15. U. Laemmli, *Nature (London)* **227**, 680 (1970).
16. Supported by NIH grants RO1 HL-28024 and AI-21301. S.T.T. was supported by National Research Service Award 1 F32 AM07477. We thank C. Brinckerhoff and C. Mainardi for helpful discussions and J. Blum for preparing rat tail collagen.
* To whom correspondence should be addressed.

7 November 1984; accepted 31 December 1984

Induction of DNA Synthesis in Cultured Rat Hepatocytes Through Stimulation of α_1 Adrenoreceptor by Norepinephrine

Abstract. *Addition of norepinephrine to primary cultures of adult rat hepatocytes stimulates the incorporation of [^{3}H]thymidine in a dose-dependent manner. This effect has been observed in serum-free medium containing epidermal growth factor and insulin. Stimulation of DNA synthesis by norepinephrine was strongly antagonized by the α_1-adrenergic antagonist prazosin but not by an α_2 antagonist or by a β-adrenergic blocker. The β agonist isoproterenol did not stimulate significant DNA synthesis. These results indicate that catecholamines interact with the α_1 adrenoreceptor to stimulate DNA synthesis in hepatocytes. Since α_1 receptors are present in most cells, this receptor may be important in cell growth regulation.*

Cell replication does not generally occur in adult mammalian hepatocytes. However, the hepatic parenchyma responds to partial resection with coordinated waves of DNA synthesis. This has made liver regeneration a useful model for the study of stimulated cell growth.

A role for catecholamines in the stimulation of hepatic DNA synthesis after partial hepatectomy has been suggested by many investigators [reviewed in (*1*)]. In general, adrenergic receptor inhibitors suppress DNA synthesis during liver regeneration in vivo, but whether this effect is mediated by α- or β-adrenergic receptors is unclear. Also unknown is whether catecholamines have a direct effect on DNA synthesis or act by indirect stimulation through increased production or release of other growth factors.

Primary cultures of parenchymal hepatocytes of high viability, capable of entering DNA synthesis after appropriate stimulation, have been established. Rat hepatocytes have been cultured in serum-supplemented or serum-free media containing epidermal growth factor (EGF) and insulin (*2, 3*). We now report that the addition of norepinephrine to cultures of rat hepatocytes results in a dose-dependent stimulation of DNA synthesis and that this effect is antagonized by prazosin, which blocks the α_1-adrenergic receptor.

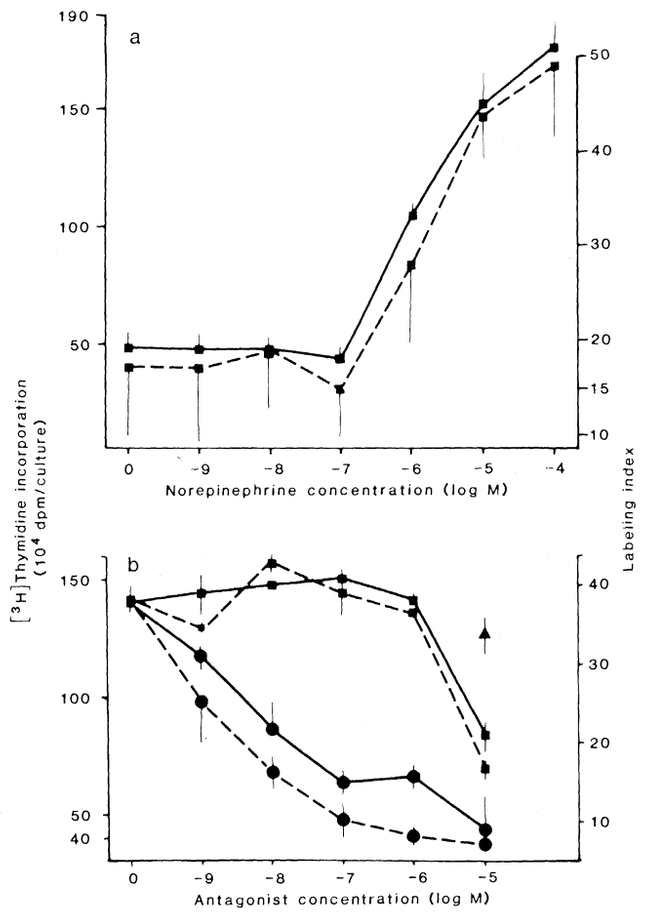
The addition of high concentrations of norepinephrine to hepatocyte cultures stimulates the incorporation of [^{3}H]thymidine into material that is precipitable with trichloroacetic acid (TCA). Incubation of cultures with various doses of norepinephrine generated the dose-response curve in Fig. 1a. To determine whether the incorporation of [^{3}H]thymidine represented a true stimulation of hepatocyte DNA synthesis, we prepared autoradiographs of parallel cultures and determined nuclear labeling indices. The results demonstrate that a dose-response relation exists between norepinephrine concentration and the stimulation of hepatocyte nuclear labeling. At $10^{-4}M$ norepinephrine, the labeling index was approximately 49 percent.

It is conceivable that toxic effects of norepinephrine might generate an apparent dose-response relation by selectively killing unlabeled cells, thereby increasing the labeling index. We therefore directly assayed the survival of the hepatocytes at all of the norepinephrine concentrations on the dose-response curve.

We used hepatocytes whose DNA had earlier been labeled with [^{3}H]thymidine and compared the amount of labeled DNA at the time of plating with the amount of labeled DNA left in the cultures at the end of the incubation with norepinephrine. No significant loss of hepatocytes was seen at any of the norepinephrine concentrations. The labeled hepatocytes were obtained from rats that had been subjected to partial hepatectomy and then given an injection of 100 μCi of [^{3}H]thymidine at 18, 24, and 36 hours after hepatectomy (*4*). The regenerated livers contained ^{3}H-labeled DNA, and any loss of cell number due to toxicity in culture could be monitored as a reduction in TCA-precipitable material per plate. Control cultures (no added norepinephrine) contained 1004 ± 78 dpm per culture after 48 hours of incubation. No concentration of norepinephrine from $10^{-9}M$ to $10^{-4}M$ caused a significant reduction in the total number of disintegrations per minute per culture ($P \geq 0.2$), indicating that there had been no loss of cells. The results in disintegrations per minute per culture (mean ± standard error of the mean) for norepinephrine at $10^{-6}M$, $10^{-5}M$, and $10^{-4}M$ were 975 ± 125, 954 ± 104, and 841 ± 49, respectively.

Insulin ($10^{-7}M$) and EGF (10 ng/ml) were present in all of the cultures used. Removal of either insulin or EGF reduced but did not abolish the response to norepinephrine. Removal of both insulin and EGF, however, eliminated the response (data not shown). Further studies are required to investigate the mode of interaction of these factors.

Adrenergic inhibitors were examined for their ability to block the stimulation induced by $10^{-5}M$ norepinephrine (Fig. 1b). Prazosin at $10^{-7}M$ reduced the nuclear labeling index to control levels. As shown in Fig. 1b, $10^{-8}M$ prazosin (an α_1 selective blocker) and $10^{-5}M$ yohimbine (an α_2 antagonist) reduced DNA synthesis to approximately the same extent. This 1000:1 ratio of effectiveness of prazosin to yohimbine is identical to that seen by Tolbert *et al.* (*5*) for the inhibition of phosphatidylinositol turnover in



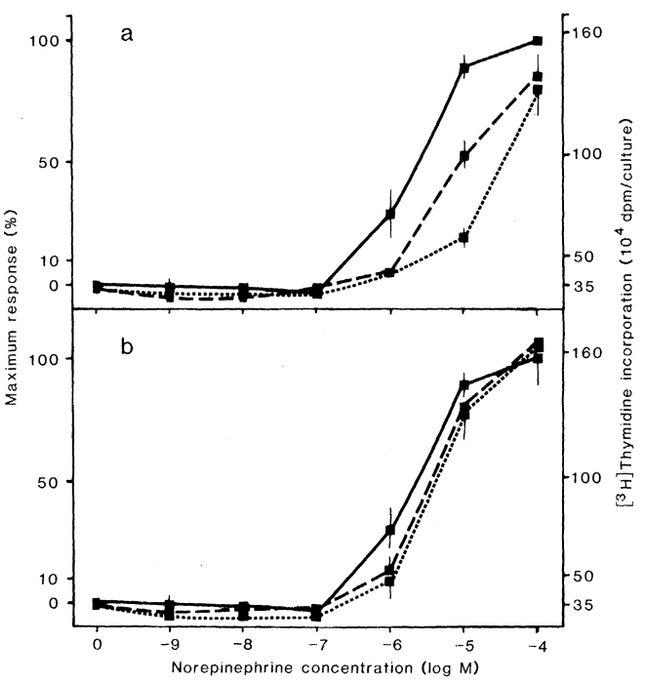


Fig. 1 (left). (a) The relation of norepinephrine concentration to hepatocyte DNA synthesis was measured by the incorporation of [^{3}H]thymidine into TCA-precipitated material (left scale, solid line); bars indicate the standard error of the mean of four independent experiments. Measured as disintegrations per minute per culture, the effect of norepinephrine is significant ($P < 0.01$) at concentrations of $10^{-6}M$ and above. The dose-response relation was also measured by autoradiographic nuclear labeling index (right scale, dashed line); bars indicate the standard error of the mean of three independent experiments. Hepatocytes were isolated from female Fisher rats (100 to 200 g) by the collagenase perfusion technique of Seglen (*11*) as modified by Michalopoulos *et al.* (*12*) and plated on collagen-coated dishes at a density of approximately 100,000 cells in 1 ml of suspension per 35-mm culture dish. Cells were allowed to settle and adhere for 2 hours in the following medium, supplemented with 5 percent fetal calf serum: minimum essential medium with Earle's salts, pyruvate (1 m*M*), aspartate (0.2 m*M*), serine (0.2 m*M*), proline (1 m*M*), gentamicin sulfate (50 μg/ml), EGF (10 ng/ml), and insulin ($10^{-7}M$). After 2 hours this was replaced by the same medium without serum, supplemented by 10-μl volumes of norepinephrine or other drugs as indicated. Cultures processed for scintillation counting of TCA-precipitable material were incubated with [^{3}H]thymidine at 5 μCi/ml; cultures processed for autoradiography contained 10 μCi/ml. All cultures were incubated for 48 hours. Labeling indices are determined as the percentage of heavily labeled hepatocyte nuclei, more than 300 nuclei being counted per culture. The processing of these cultures for autoradiography has been described (*3*). (b) Effect of increasing doses of yohimbine (filled squares) and prazosin (filled circles) on the stimulation of labeling elicited by $10^{-5}M$ norepinephrine. Norepinephrine and blockers were added simultaneously to cultures, which were incubated for 48 hours. Harvested cells were then processed for either TCA precipitation (solid lines, left scale) or autoradiography (dashed lines, right scale). A single dose of propranolol ($10^{-5}M$) is included (filled triangle); it did not significantly reduce the effect of $10^{-5}M$ norepinephrine ($P > 0.1$). Mean control values (without added norepinephrine) were 30.5×10^4 dpm per culture and 10.6 percent labeling index. Fig. 2 (right). The effects of low concentrations of (a) prazosin, an α_1-selective antagonist and (b) yohimbine, an α_2-selective blocker on the dose-response curve relating norepinephrine and DNA synthesis as measured by incorporation of [^{3}H]thymidine into TCA-precipitable material. Solid lines represent the response to norepinephrine alone; dashed lines indicate the response in the presence of $10^{-9}M$ antagonist concentration, and dotted lines show the response with $10^{-8}M$ antagonist. Bars indicate the standard error of the mean for maximum response; where bars are not visible, standard error of the mean is ≤3 percent.

isolated hepatocytes. Propranolol (a β blocker) at $10^{-5}M$ concentration had a slight effect (5 percent reduction). Yohimbine ($10^{-7}M$ to $10^{-5}M$) and prazosin ($10^{-7}M$ and $10^{-6}M$) were examined for toxic effects on labeled hepatocyte cultures. No significant loss of DNA was observed at any of the concentrations tested. The results in disintegrations per minute per culture (mean ± standard error of the mean) were control, 1004 ± 78; yohimbine ($10^{-7}M$) 1015 ± 206, ($10^{-6}M$) 902 ± 116, and ($10^{-5}M$) 941 ± 31; and prazosin ($10^{-7}M$) 988 ± 72 and ($10^{-6}M$) 901 ± 128. (For yohimbine, $P \geq 0.3$; for prazosin, $P \geq 0.5$).

The effects of lower doses ($10^{-9}M$ and $10^{-8}M$) of prazosin and yohimbine on the dose-response curve for norepinephrine are shown in Fig. 2, a and b. The ability of prazosin to shift the curve to the right is indicative of competitive inhibition for the α_1-adrenergic receptor. We have seen no significant stimulation of thymidine incorporation by clonidine, a selective α_2 agonist, or by isoproterenol, a β agonist. Epinephrine produces a dose-dependent stimulation of DNA synthesis similar to that of norepinephrine, which is also significantly inhibited by prazosin (data not shown). Liver has a high relative concentration of α_1-adrenergic receptors (*6*), and the ability of catecholamines to directly stimulate DNA synthesis through α_1 mediation suggests a significant role for these receptors in the regenerative response.

Our studies do not indicate whether the effect of norepinephrine at α_1 adrenoreceptors involves the stimulation of Ca^{2+} mobilization or phosphatidylinositol turnover. Both of these proceses occur in rat hepatocytes after the addition of norepinephrine (*5, 7*). Earlier studies (*8*) showed that vasopressin and angiotensin II also stimulate DNA synthesis in primary cultures of hepatocytes. These studies and our own indicate that at least part of the regenerative response after partial hepatectomy may be mediated by Ca^{2+} movements or phosphatidylinositol turnover, or both.

To our knowledge, this is the first evidence for regulatory effects of α_1-adrenergic receptors on DNA synthesis. Preliminary data indicate that norepinephrine is present in micromolar concentrations in the plasma of rats after partial hepatectomy, although it remains to be seen whether plasma catecholamines or those delivered by synaptic mechanisms are an important part of the regenerative stimulus. In view of the presence of the α_1-adrenergic receptor subtype in most mammalian tissues, and the potential of this receptor for the stimulation of protein kinase C (phorbol ester receptor) (*9, 10*) via diacylglycerol, the role of the α_1 receptor as a potential regulator of cell growth merits further study.

JENNIFER L. CRUISE
KEITH A. HOUCK
GEORGE K. MICHALOPOULOS*
Department of Pathology,
Duke University Medical Center,
Durham, North Carolina 27710

References and Notes

1. C. G. D. Morley and V. L. Royse, *Int. J. Biochem.* **13**, 969 (1981).
2. G. Michalopoulos *et al.*, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 1023 (1982).
3. J. A. McGowan, A. J. Strain, N. L. R. Bucher, *J. Cell. Physiol.* **108**, 353 (1981).
4. M. R. Rosenberg, S. C. Strom, G. Michalopoulos, *In Vitro* **18**, 775 (1982).
5. M. E. M. Tolbert, A. C. White, K. Aspry, J. Cutts, J. N. Fain, *J. Biol. Chem.* **255**, 1938 (1980).
6. B. B. Hoffman *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4569 (1980).
7. J. H. Exton, *Mol. Cell. Endocrinol.* **23**, 233 (1981).
8. W. E. Russell and N. L. R. Bucher, *Endocrinology* (Suppl.) **110**, 162 (1982).
9. I. B. Weinstein, *Nature (London)* **302**, 750 (1983).
10. J. F. Kuo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7039 (1980).
11. P. O. Seglen, *Methods Cell Biol.* **18**, 29 (1976).
12. G. Michalopoulos *et al.*, *Cancer Res.* **42**, 4673 (1982).
13. This work was supported by grant CA 35373 from the National Institutes of Health. J.L.C. was supported by NIH training grant T32 GM07184.
* To whom requests for reprints should be addressed.

15 June 1984; accepted 29 October 1984

Vitamin E Reversal of the Effect of Extracellular Calcium on Chemically Induced Toxicity in Hepatocytes

Abstract. *Isolated rat hepatocytes were incubated in the presence or absence of extracellular calcium and α-tocopherol succinate with three different toxic chemicals; namely, adriamycin in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea, ethyl methanesulfonate, and the calcium ionophore A23187. In the absence of extracellular calcium these three compounds were far more toxic to the cells than in its presence. The addition of vitamin E to calcium-free medium, however, protected hepatocytes against toxic injury, whereas cells incubated in medium containing calcium were not protected. Hepatocyte viability during each toxic insult correlated well with the cellular α-tocopherol content but not with the presence or absence of extracellular calcium. These results suggest that cellular α-tocopherol maintains the viability of the cell during a toxic insult and that the presence or absence of vitamin E in the incubation medium probably explains the conflicting reports on the role of extracellular calcium in toxic cell death.*

It is apparent from the numerous hypotheses that have been proposed to explain the mechanism by which chemicals produce toxic effects in biological systems (*1–3*) that this phenomenon is not well understood. One hypothesis is that chemically induced cell death is the consequence of the influx and accumulation of extracellular calcium ions in the cell (*2*). Results obtained with primary cultures of adult rat hepatocytes showed that toxic manifestations caused by various chemicals were expressed only by cells incubated with Ca^{2+} (*2*). In the absence of extracellular Ca^{2+}, cultured hepatocytes were protected from chemically induced cell death. These findings led to the hypothesis that the influx of

Table 1. α-Tocopherol content of isolated hepatocytes incubated in the presence or absence of extracellular Ca^{2+}, vitamin E, and a toxic chemical. Hepatocyte samples were obtained from the experiments described in the legend to Fig. 1. Rapid separation of viable from nonviable hepatocytes and medium was accomplished by the dibutyl phthalate centrifugation method (*18*). The concentration of α-tocopherol was measured in hexane extracts of viable hepatocytes with the use of an HPLC method (*19*). The average initial cellular α-tocopherol concentration was 0.11 ± 0.01 nmol/10^6 cells. Values represent the means and standard errors of four separate experiments.

Treatment	Cellular α-tocopherol (percentage of initial concentration)			
	Medium minus vitamin E		Medium plus vitamin E	
	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}
Control				
1 hour	74 ± 12	90 ± 7	1229 ± 147	201 ± 16
3 hours	41 ± 9	72 ± 9	2275 ± 365	672 ± 57
ADR-BCNU (4 hours)	<7	63 ± 1	870 ± 58	94 ± 6
EMS (1 hour)	13 ± 3	39 ± 9	632 ± 79	114 ± 5
A23187 (3 hours)	12 ± 4	28 ± 5	2344 ± 215	265 ± 44