and nonischemic hemisphere by using [^{3}H]naloxone and [^{3}H]dihydromorphine in untreated group A gerbils (*6*). Twenty gerbils were killed 9 hours after ligation, and the hemispheres were dissected free of the cerebellum and the brain stem and then separated. Crude synaptosomal fractions were isolated from each hemisphere, and a standard binding assay similar to that reported by Pert and Snyder (*7*) was performed. Scatchard analysis of the data indicates that there was no significant difference in either the capacity or affinity of binding to both high- and low-affinity sites between the ischemic (right) and the control (left) hemispheres.

Brains from several untreated group A gerbils were analyzed for concentrations of immunoreactive β-endorphin–like material by F. E. Bloom at the Salk Institute, La Jolla, California. Preliminary data (corrected for protein) indicate that the concentration of immunoreactive β-endorphin–like material was 40 to 80 percent higher in the ischemic right hemisphere than in the left control hemisphere.

These observations show that naloxone reverses the neurologic-deficit caused by cerebral ischemia in gerbils and that continuous administration of naloxone reduces mortality due to stroke. Our results further suggest that the action of opiates and naloxone on the neurologic deficit is stereospecific and that there appears to be no significant alteration in receptor binding capacity or the affinity of ligand binding to receptors.

Recently, Faden *et al.* (*8*) reported that systemically administered naloxone significantly prevented the development of hypotension after spinal cord injury in cats, which in turn prevented the development of extensive ischemic damage in the face of traumatic injury. Intracerebral injections of naloxone at doses much lower than those administered intraperitoneally produced the same protective effect on spinal cord function. Faden *et al.* postulated that hypotension produced by spinal cord injury may be mediated centrally and suggested the involvement of the endorphin system.

We have not been able to measure blood pressure in gerbils, although respiratory rate and thoracic excursion did not seem to be altered by either naloxone or morphine at the doses given. In several gerbils, arterial blood samples were obtained by cardiac puncture; gerbils treated with naloxone or opiate agonists had no significant alterations in arterial partial pressure of O_2, partial pressure of CO_2, or *p*H compared to untreated gerbils. In the absence of other causes, cerebral ischemia often induces systemic hypertension in animals or humans (*9*). Moreover, there were no changes in vital signs in the two patients in whom hemiplegia was reversed by naloxone (*1a*). Therefore, we do not feel that our observation is related to the pathophysiology that led to the observation of Faden *et al.*

The depressive effect of intracerebral administration of β-endorphin on motor activity is well known (*10*). On the basis of our observations in gerbils and our findings in humans, it is tempting to assume that cerebral ischemia causes an increase in the level of β-endorphin that in turn, by an unknown mechanism, produces hemiparesis. If there is no cerebral infarction, this effect can be reversed by naloxone. Our finding that naloxone did not reverse hemiplegia in a patient with focal cerebral infarction supports this hypothesis (*1a*). However, if the ischemic insult is insufficient to produce motor manifestations, administration of exogenous opiate may be sufficient to alter homeostasis and produce stroke. As shown by our results in gerbils, stroke induced by opiate agonists is a stereospecific phenomenon. The enkephalin analog Sandoz FK33824, which has potent analgesic activity in rodents (*11*), did not produce signs of stroke in group B gerbils. These observations are novel and suggest that treatment with naloxone may significantly improve functional neurologic recovery and reduce mortality after ischemic cerebral insult in humans.

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References and Notes

1. N. J. Brandt *et al.*, *N. Engl. J. Med.* **303**, 914 (1980).
1a. D. S. Baskin and Y. Hosobuchi, *Lancet* **II-1981**, 272 (1981).
2. R. Katzman, R. Clasen, I. Klatzo, J. S. Meyer, H. M. Pappins, A. G. Waltz, *Stroke* **8**, 512 (1977).
3. K. Kahn, *Neurology* **22**, 510 (1972).
4. H. M. Payan and J. R. Conard, *Stroke* **8**, 194 (1977); S. Levin and D. Sohn, *Arch. Pathol.* **87**, 315 (1969).
5. This and other injections and procedures were performed routinely 9 hours after surgery for ligation of the right common carotid artery.
6. The [^{3}H]naloxone was obtained from New England Nuclear Corporation, Boston; the [^{3}H]dihydromorphine was obtained from Amersham Corporation, Arlington Heights, Ill.
7. C. B. Pert and S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2243 (1973).
8. A. I. Faden, T. P. Jacobs, J. W. Holaday, *Science* **211**, 493 (1981).
9. T. Hayakawa, A. G. Waltz, T. Hansen, *Stroke* **8**, 426 (1977); H. H. Merritt, *A Textbook of Neurology* (Lea & Febiger, Philadelphia, ed. 5, 1973).
10. F. Bloom, D. Segal, N. Ling, R. Guillemin, *Science* **194**, 630 (1976); Y. F. Jacquet and N. Marks, *ibid.*, p. 632.
11. H. H. Roemer, R. Buescher, R. C. Hill, J. Pless, W. Bawer, F. Caidinaux, A. Caosse, D. Hauser, R. Hugnenin, *Nature (London)* **268**, 547 (1977).
12. We thank B. J. Hunter for manuscript preparation and N. Buckley for editorial assistance.

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Epinephrine Reduction of Heme: Implication for Understanding the Transmission of an Agonist Stimulus

Abstract. *Alpha-adrenergic agonists that promote platelet aggregation were found to reduce ferric heme to ferrous heme. Agents that bind iron in heme inhibited epinephrine-induced platelet aggregation. It is proposed that epinephrine first binds to its receptor and then reduces an adjacent heme group to transmit its agonist stimulus.*

The mechanism of transduction of receptor-mediated signals across biological membranes is of considerable interest (*1*). Although the platelet α-adrenergic binding site has been studied (*2*), the chemical or physical changes that transmit the agonist signal are not known. We found that epinephrine and norepinephrine can reduce ferric (Fe^{3+}) heme to ferrous (Fe^{2+}) heme (*3*). We now present evidence to suggest that epinephrine causes platelet aggregation through its ability to reduce heme.

Figure 1 shows heme reduction (*4*) by several compounds with known interactions with the platelet α-adrenergic receptor. The rank order of heme reduction (epinephrine > norepinephrine > epinine > dopamine > phenylephrine) was similar to the rank order of the same compounds as agonists (*5*). Phentolamine, which binds to the platelet receptor with high affinity (*2*) but is an inhibitor, not an agonist, was ineffective at reducing heme. Phenylephrine, which binds tightly to the platelet receptor and is an inhibitor of epinephrine aggregation but has weak agonist activity itself (*6*), caused slight but significant heme reduction at high concentrations. All com-

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pounds that reduced heme at low concentrations have two phenolic OH groups (the catechol moiety). Phenylephrine, with only one phenolic OH group, had activity only at high concentrations. Thus, the results are consistent with the catechol moiety being primarily responsible for heme reduction.

Epinine and dopamine were both less effective than epinephrine and norepinephrine, suggesting a possible role for the β-OH group in heme reduction. A role for the β-OH group to stabilize binding to Fe^{3+}-heme was considered. However, we were unable to demonstrate inhibtion of epinephrine reduction of heme by phenylethanolamine. Thus, if such an effect is present, it must be small.

Our hypothesis that epinephrine activates platelets by reducing a membrane heme group would require both binding of epinephrine to the platelet α-adrenergic receptor and heme reduction by epinephrine for cell stimulation. Thus, isoproterenol, dobutamine, and ascorbic acid, which can reduce heme (1.34 ± 0.1, 3.8 ± 0.05, and 1.7 ± 0.15, each $\times 10^{-5}$, mole of heme reduced, respectively, at concentrations of 160, 90, and 250 μM under the conditions described in the legend of Fig. 1) but do not have the appropriate structure to bind to the platelet α-adrenergic receptor (*2*), do not promote or initiate platelet aggregation (*7*). Furthermore, the inability of phentolamine, which can bind tightly to the platelet receptor, to initiate platelet aggregation can now be explained by its inability to reduce heme.

To see whether the heme group might be important to receptor binding, we studied the ability of phentolamine, phenylethylamine, and phenylethanolamine to inhibit heme reduction by epinephrine. None of these compounds, which are α-adrenergic inhibitors in platelets (*5*), had any effect on the ability of epinephrine to reduce heme. The results provide further evidence for dissociation of receptor binding and effector functions and suggest that the heme itself contributes little to receptor binding.

We propose that a molecule must have both receptor binding and effector activity to initiate aggregation. This concept is in accord with structure-activity studies of α-adrenergic agents on platelets that indicate that epinephrine is bound to its receptor primarily through interactions involving the methylamine and β-OH groups along with hydrophobic bonds involving the aromatic ring, while intrinsic agonist activity is a function of the catechol moiety (*5*). We thus agree with the suggestion by Rossi *et al.* (*5*) that binding forces orient the epinephrine molecule on the receptor to position the catechol group in the location necessary for it to interact with the component that triggers events leading to platelet aggregation. Such tight binding and positioning at an adjacent receptor may make the heme reduction by epinephrine in the platelet membrane much more efficient (that is, with a much lower absolute concentration of epinephrine) than we can demonstrate with heme solubilized in pyridine.

Table 1. Inhibition of epinephrine- and ADP-induced platelet aggregation by heme binding agents. Results are shown as mean ± S.E.M.

Agent	IC_{50} (mM)	
	Epinephrine	ADP
1,10-Phenanthroline	0.52 ± 0.13	0.93 ± 0.07*
3-Chloropyridine	0.95 ± 0.26	3.8 ± 0.4*
2,2′-Dipyridyl	0.51 ± 0.10	4.8 ± 0.25*
4,4′-Dipyridyl	0.31 ± 0.24	1.3 ± 0.3*

*$P < .05$ that the IC_{50} for epinephrine and ADP aggregation are different.

The transient, unstable, and highly active nature of reduced heme, when it is not specifically protected by a hydrophobic pocket such as exists in hemoglobin, makes reduction of heme an excellent candidate for modulating a rapid on-off process involved in enzyme activation (*3, 8*) or signal transmission across a membrane. We predict that heme in a membrane will behave similarly to heme under our artificial conditions. Indeed, this concept fits evidence that an oxidation-reduction reaction might be an important step in the initial platelet response to epinephrine (*9*).

Testing the hypothesis that epinephrine activates platelets by reducing a Fe^{3+}-heme in or near the receptor to a Fe^{2+}-heme is not an easy task since unequivocal evidence may involve isolation of a heme protein, which is only a small proportion of platelet protein (*2*). As an initial step, we studied agents that bind iron in heme to see if they inhibit epinephrine aggregation. Two types of heme binding agents, phenanthroline and compounds related to dipyridyl, were assessed.

Platelet aggregation was studied by using a Payton dual-channel aggregometer to monitor light transmission (*10*). Inhibition of aggregation to epinephrine, and for comparative purposes to adenosine diphosphate (ADP), was evaluated. In these studies, we considered only the first-wave responses. Second-wave aggregation responses reflect events such as prostaglandin synthesis that are separated by a considerable distance from transmission of the initial stimulus (*11*). It has been shown that dipyridyl and related compounds inhibit second-wave epinephrine aggregation (*12*) but, since the effect could have been solely due to inhibition of prostaglandin endoperoxide synthetase, it was essential to restudy this question. The results show that all heme liganding agents tested were effective in inhibiting first-wave epinephrine aggregation (Table 1). The effects of these heme liganding agents on epinephrine aggregation were not solely related to an overall depressant influence on platelets since the median inhibition concentrations (IC_{50}) for first-wave aggrega-

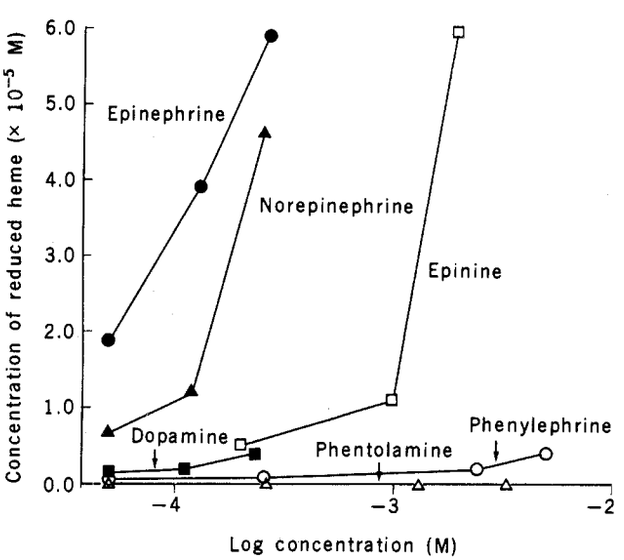

Fig. 1. Reduction of heme by epinephrine and related compounds. Each agent was added to a solution of heme in 25 percent pyridine. Reduction of heme was evaluated by measuring absorption at 558 nm. Values represent the maximum reduction for each compound at the concentration shown. Such maximum reduction occurred within the first minute after addition of the agent, except for norepinephrine, where peak reduction occurred at 2 to 3.5 minutes. Dopamine could not be tested at higher concentrations than those shown because of the formation of an interfering chromophore. Each point is the mean of triplicate determinations.

tion by epinephrine were significantly lower than those for ADP, showing some selectivity of their effect on the response to epinephrine. The unidentate binding agent 3-chloropyridine and 4,4′-dipyridyl, which has two liganding sites but is not a classical bidentate chelator, were effective in concentrations only slightly different from the bidentate chelator 2,2′-dipyridyl. Since the heme prosthetic group allows liganding to only one site on the iron by one molecule of these inhibitors (*12*), a unidentate iron-binding agent like 3-chloropyridine should be active, as indeed it was, in concentrations similar to those of 2,2′-dipyridyl. The results are therefore consistent with the concept that heme reduction is involved in the action of epinephrine on platelets.

The nature of the platelet process that might be activated by heme reduction is not known. One early event in the platelet response to epinephrine is inhibition of adenylate cyclase, but this process has been dissociated from stimulation of aggregation (*13*). Further study of the potentially important and perhaps widespread role of heme reduction in signal transmission will require better definition of this initial event.

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References and Notes

1. F. Hirata and J. Axelrod, *Science* **209**, 1082 (1980).
2. K. D. Newman, L. T. Williams, N. H. Bishopric, R. J. Lefkowitz, *J. Clin. Invest.* **61**, 395 (1978); R. W. Alexander, B. Cooper, R. I. Handin, *ibid.*, p. 1136.
3. D. A. Peterson, J. M. Gerrard, G. H. R. Rao, J. G. White, *Prostaglandins Med.* **5**, 357 (1980).
4. Heme was prepared in a 25 percent alkaline pyridine solution, *p*H 12.6, and diluted in 30 percent pyridine, *p*H 12.2, to a concentration of 0.77 or $1.54 \times 10^{-4} M$ in an ultraviolet cuvette. Heme reduction was then followed by monitoring absorption at 558 nm and quantified by $E = 30\ mM^{-1}$, where E is the extinction coefficient as described by D. A. Peterson, J. M. Gerrard, G. H. R. Rao, and J. G. White [*Prostaglandins Med.* **4**, 73 (1980)].
5. E. C. Rossi, G. Louis, E. A. Zeller, *J. Lab. Clin. Med.* **93**, 186 (1979).
6. J. A. Grant and M. C. Scrutton, *Nature (London)* **277**, 659 (1979).
7. Isoproterenol (*5*) and ascorbic acid [N. D. Goldberg, G. Graff, M. K. Haddox, J. K. Stephenson, D. B. Glass, M. E. Moser, *Adv. Cyclic Nucleotide Res.* **9**, 101 (1978)] have been tested and found to have no effect in initiating or promoting platelet aggregation. We studied dobutamine at concentrations of 5 to 100 μM and also found that it has no effect in promoting or initiating platelet aggregation.
8. D. A. Peterson and J. M. Gerrard, *Med. Hypothesis* **6**, 491 (1980).
9. J. G. White, G. H. R. Rao, J. M. Gerrard, *Am. J. Pathol.* **88**, 387 (1977).
10. G. V. R. Born and M. J. Cross, *J. Physiol. (London)* **169**, 178 (1963).
11. J. B. Smith, C. C. Ingerman, M. J. Silver, *J. Clin. Invest.* **53**, 1468 (1974); J. R. O'Brien, *Lancet* **1968-I**, 204 (1968).
12. G. H. R. Rao, A. C. Cox, J. M. Gerrard, J. G. White, *Biochim. Biophys. Acta* **628**, 468 (1980).
13. R. J. Haslam, M. M. L. Davidson, T. Davies, J. A. Lynham, M. D. McClenaghan, *Adv. Cyclic Nucleotide Res.* **9**, 533 (1978); M. L. Steer and E. W. Salzman, *ibid.* **12**, 71 (1980).
14. We are grateful for financial support for these studies from USPHS grant HL 11880, grant MA7396 from the Canadian MRC, and a grant from the Children's Hospital of Winnipeg Research Foundation. J.M.G. is the recipient of a Canadian MRC scholarship. Send reprint requests and correspondence to J.M.G.

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Preferential Attack of Mitochondrial DNA by Aflatoxin B_1 During Hepatocarcinogenesis

Abstract. *Administration of the hepatic carcinogen aflatoxin B_1 to experimental animals results in covalent binding to liver mitochondrial DNA at concentrations three to four times higher than nuclear DNA. The concentration of carcinogen adducts in mitochondrial DNA remains unchanged even after 24 hours, possibly because of lack of excision repair. Similarly, mitochondrial transcription and translation remain inhibited up to 24 hours suggesting long-term effects of aflatoxin B_1 on the mitochondrial genetic system.*

Mitochondria from tumor cells have altered functional and structural properties (*1*). It is not known if these abnormalities are due to altered mitochondrial genetic systems or indirect effects involving the nuclear genes. Recent studies in our laboratory showed the presence of a cytochrome P-450 type of monooxygenase system in rat liver mitochondria capable of activating the hepatic carcinogen aflatoxin B_1 (AFB_1) into an electrophilic reactive form which then covalently modifies mitochondrial DNA, RNA, and protein (*2*). Activated metabolites of benzo[*a*]pyrene and other polycyclic aromatic hydrocarbons (*3*) incubated with tissue culture cells cause substantially more modification of mitochondrial DNA than of nuclear DNA. We now report that AFB_1 administered to experimental animals covalently binds to liver mitochondrial DNA more than to nuclear DNA. The chemical modification of mitochondrial DNA persists even after 24 hours and is accompanied by pronounced inhibition of mitochondrial RNA and protein synthetic activities.

When AFB_1 is administered intraperitoneally to rats, the amount of carcinogen in the hepatic tissue increases rapidly to reach a peak in about 2 to 3 hours with 15 to 18 percent of the drug dose localized in this tissue (*4*). At this time, nearly 60 percent of hepatic AFB_1 is in covalently bound form. Table 1 shows that at a peak time of 3 hours almost 24 percent of the bound AFB_1 is associated with mitochondria whereas 22 percent is

Table 1. Intracellular distribution of AFB_1 in hepatic tissue. Male Sprague-Dawley rats (150 to 180 g) were injected intraperitoneally with 30 μCi of [^{3}H]AFB_1 (30 μCi/2.9 μmole) to yield a dose rate of 6 mg/kg. At intervals, the rats were killed and the livers were removed, washed free of blood clots, minced, and homogenized in 2 m*M* Hepes (*p*H 7.5), 70 m*M* sucrose, 220 m*M* D-mannitol, 1 m*M* EDTA, and 0.5 g of bovine serum albumin per liter as described (*2*). The homogenate was adjusted to 10 percent (weight to volume) with the above buffer and used for isolating the nuclear fraction (the pellet obtained at 1100 *g*), mitochondrial fraction (the pellet obtained at 8500*g*), and postmitochondrial supernatant as described (*2, 10*). The nuclear pellet was washed twice with buffer containing 20 m*M* tris-HCl (*p*H 7.5), 60 m*M* KCl, 2 m*M* $CaCl_2$, and 0.5 percent Triton X-100 to remove contaminating membranes (*12*); the mitochondrial pellet was washed with digitonin (0.1 mg per milligram of protein) to remove the outer membrane (*13*), and the soluble fractions from these washings were included in the postmitochondrial supernatant. The amount of bound [^{3}H]AFB_1 in each fraction was assayed by precipitation with cold CCl_3COOH as described (*2*). Data from three independent estimates were used for calculating the mean and standard deviations. Values in parentheses are percentages of the total cellular bound [^{3}H]AFB_1 at the specified time points.

Cell fraction	Amount of [^{3}H]AFB_1 bound (nmole)		
	At 3 hours	At 12 hours	At 24 hours
Total intracellular	199.0 ± 12.1 (100)	127.0 ± 4.2 (100)	56.0 ± 3.2 (100)
Post mitochondrial supernatant	109.0 ± 8.1 (54.4)	67.5 ± 2.1 (53.4)	22.9 ± 2.9 (38.2)
Mitoplasts	49.6 ± 4.1 (23.5)	36.5 ± 4.1 (28.7)	27.1 ± 4.8 (49.6)
Nucleus	44.1 ± 4.6 (22.1)	20.9 ± 2.2 (15.0)	6.4 ± 0.77 (12.1)

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