Cyclic Oxidation-Reduction Reactions Regulate Thromboxane A2/Prostaglandin H2 Receptor Number and Affinity in Human Platelet Membranes*

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The radiolabeled thromboxane A2/prostaglandin H2 (TXA2/PGH2) agonist [125I]-BOP bound to the TXA2/PGH2 receptor on human platelet membranes. Scatchard analysis showed that pretreatment of platelet membranes with the reducing agent dithiothreitol (DTT) (10 mM) for 10 min decreased maximal [125I]-BOP binding (Bmax) from 1.51 ± 0.11 pmol/mg to 0.51 ± 0.05 pmol/mg (p = 0.001) and increased the affinity of the remaining binding sites (Kd = 647 ± 64 pm (untreated), 363 ± 46 pm (treated), p = 0.006). Prolonged incubation of membranes with DTT (10 mM) for 40 min further reduced the Bmax to 0.23 ± 0.08 pmol/mg (p = 0.001 from untreated), and the binding affinity remained elevated (Kd = 334 ± 117 pm, p = 0.035 from untreated). Kinetic analysis of [125I]-BOP binding indicated that the apparent increase in binding affinity after DTT treatment was due exclusively to an increase in dissociation rate. The effects of DTT on [125I]-BOP binding were dose-dependent with an EC50 of 8.1 ± 0.2 mM. DTT inactivation of TXA2/PGH2 receptors was time-dependent with a second order rate constant (k2) of 0.123 m-1 s-1 at 20 °C.

The platelet membrane [125I]-BOP binding site was partially protected from DTT inactivation by prior occupation with the ligand. TXA2/PGH2 receptor protection by I-BOP was dose-dependent and linearly related (r = 0.97, p = 0.002) to the proportion of receptors occupied, but was incomplete since agonist occupation of 89% of the total number of receptors resulted in only a 38% protective effect.

Inhibition of [125I]-BOP binding after reduction with DTT could be made permanent by addition of the sulfhydryl alkylating agent N-ethylmaleimide (25 mM), but was completely reversed by reoxidation with dithionitrobenzoic acid (DTNB) (5 mM). Oxidation of untreated receptors with DTNB resulted in a 64% increase in [125I]-BOP binding sites from 1.65 ± 0.12 pmol/mg to 2.70 ± 0.08 pmol/mg (p = 0.013) without affecting binding affinity. DTNB-induced increases in [125I]-BOP binding were concentration-dependent with an EC50 of 688 ± 106 μM and occurred in less than 1 min at 37 °C.

In the absence of DTT, alkylation of free sulfhydryl groups with N-ethylmaleimide reduced [125I]-BOP Bmax in platelet membranes to 0.85 ± 0.08 pmol/mg (p = 0.003), but did not change the affinity of the remaining receptors. The EC50 for N-ethylmaleimide inactivation of TXA2/PGH2 receptors was 139 ± 8 mM, and the k2 in time course experiments was 0.067 m-1 s-1 at 20 °C.

Collectively, these data indicate that the human platelet TXA2/PGH2 receptor possesses disulfide bond(s) and sulfhydryl group(s) located at or near the ligand binding site. The abilities of oxidizing and reducing agents to respectively up and down regulate the density of receptors strongly suggest that the membrane receptor exists in variable redox states and that cyclic disulfide-sulfhydryl interchange reactions are a possible mechanism for acute reversible heterospecific regulation of TXA2/PGH2 receptors in human platelets.

Platelet thromboxane A2/prostaglandin H2 (TXA2/PGH2) receptors translate the binding of TXA2, PGH2, or their stable analogs into the cellular responses of increased free calcium concentration and myosin light chain phosphorylation which, in turn, culminate in platelet shape change, adhesion, dense granule secretion, and aggregation (1, 2). The structure/function relationships of the TXA2/PGH2 receptor necessary to mediate these phenomena are unclear. Recently, attention has been focused on the processes, but not the mechanisms, of acute regulation of human platelet TXA2/PGH2 receptor affinity and number (3, 4). In theory, homologous and heterologous regulation of receptors may be achieved via covalent or noncovalent bond formation at the receptor protein itself or in associated regulatory proteins. Examples of known mechanisms of covalent receptor modification include receptor phosphorylation (5–7), proteolysis (8, 9), and disulfide-sulfhydryl exchange reactions (10–13).

The disulfide bond reducing agent dithiothreitol (DTT) (14) has been employed to investigate the effects of disulfide reduction on the function of various membrane receptors. The effects of DTT on the acetylcholine receptor of eel electroplax indicated the presence of a disulfide bond whose reduction alters the specificity of the receptor for cholinergic agonists and antagonists (10). In human placental membranes, DTT1 converted the insulin receptor to a low affinity form and

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1 The abbreviations used are: TXA2, thromboxane A2; PGH2, prostaglandin H2; I-BOP, [15-(4-azido-2-benzyl)-3-oxabicyclo[2.2.1]hept-2-y]-5-heptenoic acid; U46619, 15S-hydroxy-11α,9α(epoxymethano)prosta-5Z,13E-dienoic acid; SQ29,548, [(15S,18Z)-5,9,13-trienoic acid]-1-[2-(phenylamino)carbonyl]-hydrazino[methyl] 7 oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Platelet Thromboxane A2 Receptor Regulation

...decreased total binding (11), while DTT increased binding to the insulin receptor in adipocyte membranes (15). Finally, DTT reduction of disulfide bonds in \( \beta \)-adrenergic receptors has been shown to decrease ligand binding without changing receptor affinity (12) and to functionally activate the receptor (16). Modification of free sulfhydryl groups at the binding site of canine cardiac \( \beta \)-adrenergic receptors (13) and in the guanyl nucleotide binding protein associated with the \( \beta \)-adrenergic receptor of turkey erythrocyte membranes (17) may also affect receptor function.

In the present study, the possibility that disulfide-sulfhydryl reactions play a role in the regulation of human platelet TXA2/PGH2 receptors was tested by examining the effects of the reducing agent DTT, the oxidizing agent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and the sulfhydryl alkylating agent N-ethylmaleimide (NEM) on binding of the radioligand TXA2/PGH2 agonist \( \cdot I-BOP \) (18) to human platelet membranes. Sulphhydryl oxidation acutely increased the number of membrane receptors without affecting binding affinity. Conversely, disulfide bond reduction reversibly decreased the total number of membrane receptors and increased the agonist affinity of the remaining receptors.

**EXPERIMENTAL PROCEDURES**

The following were obtained as gifts: \( \cdot I-BOP \), the optically active precursor for \( \cdot I-BOP \) (Dr. Dale Mais and Perry Halushka, Medical University of South Carolina, Charleston, SC), and SQ29,548 (19) (Dr. Martin Ogletree, Squibb Institute for Medical Research, Princeton, NJ). Dithiobisreitol (DTP) was purchased from Bio-Rad. N-Ethylmaleimide, dithiothreitol (DTNB), and sulfhydryl alkylating agent N-ethylmaleimide (NEM) on binding of the radioligand TXA2/PGH2 agonist \( \cdot I-BOP \) (18) to human platelet membranes. Sulphhydryl oxidation acutely increased the number of membrane receptors without affecting binding affinity. Conversely, disulfide bond reduction reversibly decreased the total number of membrane receptors and increased the agonist affinity of the remaining receptors.

For studies of solubilized TXA2/PGH2 receptor, the prepared platelet membranes (25 mg/ml) were solubilized by the addition of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and the sulfhydryl alkylating agent N-ethylmaleimide (NEM) on binding of the radioligand TXA2/PGH2 agonist \( \cdot I-BOP \) (18) to human platelet membranes. Sulphhydryl oxidation acutely increased the number of membrane receptors without affecting binding affinity. Conversely, disulfide bond reduction reversibly decreased the total number of membrane receptors and increased the agonist affinity of the remaining receptors.

**RESULTS**

**Binding of \( \cdot I-BOP \) to Human Platelet Membranes—**\( \cdot I-BOP \) was fully displaced from its binding site on crude human platelet membranes by the unlabeled TXA2/PGH2 agonist \( \cdot I-BOP \) and US46619 (Fig. 1). The IC50 for I-BOP was 560 ± 29 pm with a pseudo-Hill coefficient of -0.67 ± 0.01. The IC50 for US46619 was 11 ± 0.7 pm with a pseudo-Hill coefficient of -0.61 ± 0.02. In eight membrane preparations, nonlinear computerized analysis showed that the I-BOP competition data would fit to either a one- or two-site model, but that the single site fit was statistically superior. The resulting single site IC50 = 647 ± 64 pm with a binding capacity of 1.51 ± 0.11 pmol/mg of platelet protein (Fig. 2). For a two-site model, the IC50 values were 77 ± 8 pm and 8.0 ± 0.7 nm with the high affinity site comprising 7% of the total number of binding sites.

**Effect of Dithiobisreitol on TXA2/PGH2 Receptors—**Specific binding of \( \cdot I-BOP \) to human platelet TXA2/PGH2 receptors was inhibited 42% by preincubating platelet membranes with 10 mM DTT for 10 min (Table I). When the disulfide reducing action of DTT was not quenched after 10 min with the sulfhydryl alkylating agent N-ethylmaleimide, a maximum of 76% inhibition of specific ligand binding occurred (Table I). If added simultaneously to membranes in a 1:2.5 molar ratio, DTT and NEM quenched each other and did not affect binding (Table I). When DTT reduced membranes were reoxidized with DTNB prior to the binding assay, 95% of the lost...
**Platelet Thromboxane A2 Receptor Regulation**

**Fig. 1.** Displacement of \(^{125}\text{I}-\text{BOP}\) from human platelet membranes by \(\text{TXAZ/PGHz mimetics:}^{125}\text{I}-\text{BOP (squares)}\) and \(\text{U46619 (triangles).}\) Each point represents the means ± S.E. of eight \(\text{I}-\text{BOP}\) or four \(\text{U46619}\) displacement experiments performed with duplicate determinations as described under "Experimental Procedures."

**Fig. 2.** Effects of oxidation and reduction on \(^{125}\text{I}-\text{BOP binding to platelet membranes.}\) Equilibrium binding was performed as described under "Experimental Procedures," and the data were analyzed by the method of Scatchard using the Ligand program (22). Untreated membranes \((n = 8)\) are shown as open squares, membranes oxidized with \(5 \text{mM dithionitrobenzoate}\) \((n = 4)\) are closed squares, and membranes reduced with \(10 \text{mM dithiothreitol}\) \((n = 6)\) are closed triangles.

binding activity was restored (Table II). However, no restoration of binding activity was observed when reoxidation was prevented by alkylation of sulphydryl groups with \(25 \text{mM NEM}\) in the wash buffer. Therefore, in the ensuing experiments, DTT was quenched, and reoxidation of DTT-reduced disulfide bonds was avoided by the addition of NEM at 2.5 times the molar concentration of DTT.

The possibility that the binding changes observed after DTT treatment were caused by alterations in the orientation of \(\text{TXAZ/PGHz receptors within platelet membranes was investigated by solubilizing the receptor with the detergent CHAPS. Suspended in detergent micelles, the receptor does not maintain a specific orientation to other membrane proteins. As shown in Table I, DTT decreased \(^{125}\text{I}-\text{BOP binding to solubilized membranes in a manner identical with that seen in crude membranes. Thus, the action of DTT appears to be specific to the TXAZ/PGHz receptor rather than nonspecifically affecting membrane structure.}

Inhibition of \(^{125}\text{I}-\text{BOP binding was dependent on the concentration of DTT (Fig. 3). The EC}_{50} \text{ for binding inhibition was } 8.1 ± 0.2 \text{ mM. The threshold for binding inhibition was } 1 \text{ mM, and virtually all specific binding was abolished at a DTT concentration of } 100 \text{ mM for } 10 \text{ min. In subsequent experiments, platelet membranes were preincubated with } 10 \text{ mM DTT, a concentration approximating the EC}_{50}. \text{ In this manner, sufficient binding activity remained after DTT treatment}

### Table I

Inhibition of \(^{125}\text{I}-\text{BOP binding to human platelet membranes by pretreatment with reducing and alkylating agents}

<table>
<thead>
<tr>
<th>Reagent added</th>
<th>(^{125}\text{I}-\text{BOP specifically bound (cpm/tube)}</th>
<th>n</th>
<th>p (from control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude platelet membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7988 ± 792</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DTT (10 min)</td>
<td>4648 ± 647</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>DTT (40 min)</td>
<td>1912 ± 118</td>
<td>3</td>
<td>0.001</td>
</tr>
<tr>
<td>NEM</td>
<td>3898 ± 421</td>
<td>5</td>
<td>0.002</td>
</tr>
<tr>
<td>DTT + NEM</td>
<td>7292 ± 1117</td>
<td>3</td>
<td>NS*</td>
</tr>
<tr>
<td>Solubilized platelet membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>871</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>DTT (10 min)</td>
<td>201</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>DTT (40 min)</td>
<td>162</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*NS, not significant.

### Table II

Augmentation of \(^{125}\text{I}-\text{BOP binding to human platelet membranes by oxidation with dithionitrobenzoic acid}

<table>
<thead>
<tr>
<th>Reagent added</th>
<th>(^{125}\text{I}-\text{BOP specifically bound (cpm/0.1 mg)}</th>
<th>n</th>
<th>p (from control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7880 ± 246</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>DTTN (5 mM)</td>
<td>3996 ± 614</td>
<td>4</td>
<td>0.04</td>
</tr>
<tr>
<td>DTT/DTNB</td>
<td>7602 ± 1480</td>
<td>3</td>
<td>NS*</td>
</tr>
<tr>
<td>DTT/NEM/ DTNB</td>
<td>3418 ± 648</td>
<td>3</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Scatchard analysis

<table>
<thead>
<tr>
<th>Reagent added</th>
<th>Binding parameters (p value, paired t)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(K_d ) (pM) (pmol/mg)</td>
<td>4</td>
</tr>
<tr>
<td>DTTN (5 mM)</td>
<td>872 ± 65</td>
<td>1.65 ± 0.12</td>
</tr>
<tr>
<td>(NS)</td>
<td>912 ± 60</td>
<td>2.70 ± 0.08</td>
</tr>
</tbody>
</table>

*NS, not significant.
Fig. 3. Inactivation of TXA₂/PGH₂ receptors as a function of DTT concentration. Membranes were preincubated with increasing concentrations of DTT for 10 min as described under “Experimental Procedures.” The ratio of binding after DTT treatment to control binding (B/R₀) is shown as a function of concentration of DTT. Each point represents the mean ± S.E. of four experiments performed with duplicate determinations.

TABLE III

Decreased TXA₂/PGH₂ receptor binding capacity and increased binding affinity for agonists in membranes treated with dithiothreitol

<table>
<thead>
<tr>
<th>Reagent added</th>
<th>Binding parameters (p value)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>¹²⁵I-BOP displacement</td>
<td>Kᵦ</td>
<td>Bₘₐₓ</td>
</tr>
<tr>
<td>Control</td>
<td>647 ± 64</td>
<td>1.51 ± 0.11</td>
</tr>
<tr>
<td>DTT (10 min)</td>
<td>363 ± 46 (0.006)</td>
<td>0.51 ± 0.05 (0.001)</td>
</tr>
<tr>
<td>DTT (40 min)</td>
<td>334 ± 117 (0.035)</td>
<td>0.23 ± 0.08 (0.001)</td>
</tr>
<tr>
<td>NEM (40 min)</td>
<td>655 ± 90 (NS)²</td>
<td>0.35 ± 0.08 (0.000)</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Specifically bound cpm</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>11 ± 0.7</td>
<td>6117 ± 133</td>
</tr>
<tr>
<td>DTT (10 min)</td>
<td>8 ± 0.4 (0.012)</td>
<td>3273 ± 236 (0.001)</td>
</tr>
</tbody>
</table>

For examination of the binding characteristics of unincubated receptors.

After treatment with DTT, Scatchard analysis of binding isotherms (Fig. 2) revealed that the inhibition of specific binding was due to a decline in the total number of TXA₂/PGH₂ receptors present. Ten-min exposure of membranes to DTT (10 mM) decreased ¹²⁵I-BOP Bₘₐₓ by 66%, and a 40-min exposure reduced binding capacity by 85%. However, after both the 10-min and 40-min exposure to DTT, there was an approximately 2-fold increase in the affinity of the remaining receptors (Table III). Although it was not statistically superior to a single site model, the binding data from DTT-treated membranes, like untreated membranes, could be fit to a two-site model. Analyzed in this fashion, the Kᵦ values in DTT-treated membranes were 63 ± 9 pM and 9.9 ± 1.3 nM with the higher affinity site comprising 6% of the total binding. Changes in the binding Kᵦ for ¹²⁵I-BOP after disulfide reduction of platelet membranes were also measured by determining the ligand association (kₐ) and dissociation (kᵦ) rate constants (Fig. 4). The kinetically determined Kᵦ (kₐ/kᵦ) in untreated platelet membranes was 451 ± 120 pM, and, after treatment with 10 mM DTT for 10 min, was 62 ± 25 pM (n = 5 each, p = 0.015). The decrease in Kᵦ appeared to result exclusively from an increase in the rate of association of ¹²⁵I-BOP to the receptor (kₐ = 9.1 ± 2.9 × 10⁸ M⁻¹ min⁻¹ untreated, 5.8 ± 0.4 × 10⁸ M⁻¹ min⁻¹ DTT-treated, p = 0.001) with no apparent change in the rate of ligand dissociation (kᵦ = 0.028 ± 0.001 min⁻¹ untreated, 0.033 ± 0.012 min⁻¹ DTT-treated, p = NS).

Incubation of membranes with DTT also decreased the amount of ¹²⁵I-BOP binding in U46619 displacement studies (Table III). After DTT treatment, the IC₅₀ for U46619 competition of ¹²⁵I-BOP was slightly, but significantly, decreased (Table III), again indicating increased affinity of DTT-inactivated receptors for TXA₂/PGH₂ agonists. The pseudo-Hill coefficient after DTT treatment (−0.56 ± 0.02) did not differ from that in untreated membranes.

Inhibition of ¹²⁵I-BOP binding by 10 mM DTT was exponentially related to the preincubation time (Fig. 5) with a half-time of 6.5 min. Approximately 50% of the total binding was sensitive to DTT at this concentration. When the proportion of DTT-sensitive binding sites was plotted on a logarithmic scale, the time course of receptor inactivation was linear (Fig. 5, inset). Since the concentration of DTT (10 mM) greatly exceeded the concentration of receptors (0.015 nM), the reaction followed pseudo-first order kinetics, and the pseudo-first order rate constant (kₜ) was given by the absolute value of the slope of the natural log transformed time curve (Fig. 5, inset). At a DTT concentration of 10 mM, kₜ equals 0.074 min⁻¹. The second order rate constant (kₜ) of the reaction is defined as kₜ/DDT; therefore, kₜ is equal to 0.123 M⁻¹ min⁻¹. The second order rate constant (kₜ) of the reaction is defined as kₜ/DDT; therefore, kₜ is equal to 0.123 M⁻¹ min⁻¹. Protection of TXA₂/PGH₂ Receptors by I-BOP—To further establish that disulfide reduction specifically perturbed the TXA₂/PGH₂ receptor, the actions of DTT were blocked by preincubating membranes with ¹²⁵I-BOP. Occupation of receptors by the ligand conferred a concentration-dependent protective effect against inactivation by DTT. When receptor protection was plotted as a function of ¹²⁵I-BOP concentration, the result was a shallow hyperbola (Fig. 6). Since, at high concentrations of radioactive ligand ([L*]), the concentration of receptor occupied by ligand ([R-L*]) is given by:

\[ [R-L*] = \frac{[R]_t}{1 + K_a/[L*]} \]

where \([R]_t\) is the total concentration of receptors (1.51 pmol/mg) and Kᵦ is the dissociation constant of the ligand for the receptor (647 pM), protection was plotted as a function of receptor occupation at the different concentrations of added ligand. The relationship of receptor occupation to protection was linear (Fig. 6, inset), and extrapolation of this line to 100% receptor occupancy revealed that the maximal protection afforded by the ligand was 38%. Therefore, when all of the binding sites are occupied by ligand, incubation of the membranes with 10 mM I-BOP for 10 min results in 89% of the total receptors remaining functional compared to 70% when no ligand is present. Comparative analysis of these data (as in Fig. 5, inset) indicates that I-BOP inactivation of occupied receptors occurs with a kₜ of 0.024 min⁻¹ or at one-third the rate of inactivation of unoccupied receptors.

Effects of Dithionitrobenzoate on TXA₂/PGH₂ Receptors—
FIG. 4. Kinetics of $^{125}$I-BOP binding to platelet membranes. Time courses of association of $^{125}$I-BOP to untreated (a) or DTT-treated (10 mM, 10 min) (b) platelet membranes. Each point is the mean ± S.E. of five experiments performed with duplicate determinations. $^{125}$I-BOP concentrations used were 50 pm (squares), 200 pm (diamonds), and 500 pm (triangles). The data were analyzed assuming a pseudo-first order process, and their slopes ($k_{bobs}$) were plotted as a function of molar $^{125}$I-BOP concentrations (c). The slopes of these lines (closed figures = control, open figures = DTT-treated) are the association rate constants ($k_{a}$) and the y axis-intercepts are the dissociation rate constants ($k_{d}$). The kinetically derived $K_d$ values are given by $k_{d}/k_{a}$ (see text).

FIG. 5. Time course of TXA$_2$/PGH$_2$ receptor inactivation by DTT. Membranes were preincubated with 10 mM DTT for various periods of time and quenched with 25 mM NEM as described under "Experimental Procedures." The ratio of DTT-sensitive specific binding is represented as a function of preincubation time. Each point represents the mean of three experiments performed in duplicate. The S.E. for these data were too small to graphically demonstrate. The inset shows a logarithmic transformation of the time course data. The absolute value of the slope gives the observed rate constant or $K_{obs}$ which was 0.074 m$^{-1}$.

FIG. 6. Protection of TXA$_2$/PGH$_2$ receptors by I-BOP. Platelet membranes were preincubated with increasing concentrations of $^{125}$I-BOP prior to addition of DTT as described under "Experimental Procedures." The relationship of $^{125}$I-BOP concentration to protective effect is shown. Each point represents the means ± S.E. of three experiments performed in duplicate. The inset shows the same data expressed as a function of the fraction of TXA$_2$/PGH$_2$ receptors occupied. The line was drawn by linear regression analysis of unweighted data points ($p < 0.01, r = 0.97$).

Specific binding of $^{125}$I-BOP to human platelet TXA$_2$/PGH$_2$ receptors was augmented 27% ($p = 0.04$) by oxidation with DTNB (5 mM) (Table II). Augmentation of $^{125}$I-BOP binding was concentration-dependent with an $EC_{50}$ of 668 ± 106 µM (Fig. 7). Scatchard analysis (Fig. 2) of the binding isotherms demonstrated that the increase in specific binding resulted from a 64% increase in binding capacity for the ligand (Table II). The affinity of $^{125}$I-BOP for the receptor was not altered by oxidation (Table II).

Analysis of the time course of augmentation of $^{125}$I-BOP binding by 5 mM DTNB at 37 °C and 10 °C revealed that the reaction was complete within 1 to 2 min. Therefore, the rate constant could not be accurately determined.

Effects of N-Ethylmaleimide on TXA$_2$/PGH$_2$ Receptors—To
confirm that free sulfhydryl groups, which could form a substrate for oxidation to disulfide bonds by DTNB, were present at or near the platelet TXA2/PGH2 receptor. The effects of alkylation on sulfhydryl groups with NEM were characterized. Specific 125I-BOP binding to platelet TXA2/PGH2 receptors was inhibited ($p = 0.002$, Table I) by NEM in a concentration-dependent manner (Fig. 8, upper) with an EC$_{50}$ of 139 ± 8 nM. The minimal effective concentration of NEM was 25 mM, and nearly 25% of control specific binding remained after incubation of membranes with 500 mM NEM for 40 min.

Scatchard plots of NEM-treated membranes revealed a 44% decrease in the total number of TXA2/PGH2 receptors without any change in the affinity of the remaining sites (Table III). As noted previously and in Table I, simultaneous addition of DTT and NEM in a 1:2.5 molar ratio abolished any effects on 125I-BOP binding to platelet membranes.

Inactivation of 125I-BOP binding by NEM was time-dependent with a half-time of 12 min (Fig. 8, lower). A logarithmic replot of the time inactivation data was linear ($y = 0.131 - 0.101x, r = 0.97$) giving a second order rate constant of 0.067 M$^{-1}$ s$^{-1}$.

The possibility that these agents were altering 125I-BOP binding by changing the reaction pH was tested. DTT (10 mM) and NEM (25 mM) did not change the final pH in the binding studies. DTNB (5 mM) decreased the final pH to 7.0. We have previously shown that 125I-BOP binding to platelet membranes is stable from pH 4.8 to 8.0.$^2$ Thus, pH changes cannot explain the present findings.

**DISCUSSION**

125I-BOP binds to functional TXA2/PGH2 receptor(s) on human platelets (18, 24). In this study, oxidation and reduction of sulfhydryl/disulfide groups was found to acutely and reversibly regulate TXA2/PGH2 receptor number as assessed by 125I-BOP binding to human platelet membranes. Preincubation of platelet membranes with DTT resulted in a concentration-dependent decrease in the total number of 125I-BOP binding sites. The affinity of I-BOP and U46619 for the remaining binding sites was significantly increased. Reduction by DTT of one or several receptor-associated disulfide bonds is the likely explanation for the observed effects since: 1) the inhibitory effect of DTT on 125I-BOP binding was completely reversed by reoxidation of sulfhydryl groups with DTNB, and 2) alkylation of free sulfhydryl groups with NEM made the effects of DTT irreversible.

Prior occupation of the receptor binding site by 125I-BOP partially protected the receptor against disulfide bond reduction by DTT. Extrapolation of the receptor occupancy/protection relationship in Fig. 6 indicated that the inhibitory effect of DTT on 125I-BOP binding was reduced by 38% when all of the available receptors were occupied by ligand. Comparison of the rate constants for DTT inhibition of binding for ligand-occupied receptor and unoccupied receptor reveals that DTT inactivation occurred 3 times faster in free receptors compared to occupied receptors. The incomplete protective effect that receptor occupation affords indicates that protection may not result from physical obstruction, by the ligand, of an essential disulfide bond at the ligand binding site. Instead, conformational changes in the receptor molecule resulting from binding

$^2$ G. W. Dorn II, unpublished observations.
of the agonist ligand may "hide" one or more disulfide bonds, thus slowing the rate of DTT inactivation. However, shielding, by I-BOP, of an essential disulfide bond located at or near the ligand binding site cannot be excluded as the protective mechanism.

Inhibition of ligand binding by DTT has been reported for several other receptors. Reduction by DTT of cholinergic receptors on eel electroplax altered the specificity of the preparation for cholinergic agents. These effects could be reversed by reoxidation with DTNB, but alkylation of thiol groups after DTT treatment prevented the reversal (10). In placental membranes, disulfide reduction of insulin receptors with DTT resulted in decreased total binding with conversion of the receptor to a lower affinity form by abolishing the usually observed high affinity portion of the Scatchard plot (11). Diithiothreitol also alters the structure and function of the β-adrenergic receptor. Treatment with DTT inhibited the response of guinea pig atria to β-adrenergic agonists (25) and reversibly inhibited [3H]dihydroalprenolol binding to turkey erythrocyte membranes (12). In the latter study, as in the present study, binding activity was protected by agonist and antagonist occupation of the receptor, and the DTT effect was made irreversible by sulfhydryl alkylation. In C6 glioma cell membranes, DTT reversibly decreased the affinity of the receptor for β-adrenergic ligands without changing the number of binding sites (26).

In view of these, and other, examples of receptor modification by disulfide bond reduction, the observation that DTT inactivates human platelet TXAZ/PGHZ receptors is novel but somewhat predictable. However, finding that apparent receptor affinity for agonists increased in uninactivated receptors after DTT treatment was unexpected. There are several possible explanations for this finding: 1) a homogenous receptor population exists in differing redox states such that disulfide reduction increases the overall apparent affinity of the receptor; 2) two separate receptors exist having different affinities; the lower affinity receptor appearing to be more sensitive to reduction by DTT and inactivated more rapidly, thereby leaving a greater proportion of uninactivated high affinity receptors after DTT treatment; 3) no actual change in receptor affinity occurs after DTT treatment and the results are an analytical aberration. The present study cannot distinguish between the first two possibilities. However, the third possibility is less likely since, in separate experiments, the kinetically derived Kd for 125I-BOP also decreased after DTT treatment as did the IC50 for the TXAZ/PGHZ agonist U46619. The decrease in Kd after DTT treatment in the kinetic studies was totally due to a faster rate of ligand-receptor association, while the rate of ligand-receptor dissociation remained constant. This surprising finding suggests that disulfide bond reduction facilitates diffusion of ligand into the binding site, perhaps by altering the tertiary structure of the receptor or other closely associated proteins and physically "unblocking" access to the binding site.

Since the sulfhydryl alkylation reagent N-ethylmaleimide inhibited 125I-BOP binding to untreated platelet membranes without changing the affinity of uninactivated receptors, it appears that a free sulfhydryl group exists on the human platelet TXAZ/PGHZ receptor. An unexpected finding of this study was that oxidation of untreated platelet membranes, and presumably formation of new disulfide bonds, rapidly increased the number of 125I-BOP binding sites to 164% of control values. These results, together with the inhibitory effects of disulfide reduction and sulfhydryl alkylation, suggest that the native human platelet TXAZ/PGHZ receptor possesses both disulfide and sulfhydryl groups and exists in two states, reduced (inactive) and oxidized (active). The oxidized-active state is the predominant native form comprising approximately two-thirds of the total number of receptors. One can speculate that, under varying physiologic conditions of oxidation and reduction, heterospecific regulation of platelet TXAZ/PGHZ receptor number can occur via cyclic disulfide-sulfhydryl interchange reactions. A teleologic rationale for such a disulfide-sulfhydryl redox cycle would apply in situations of hemorrhage and tissue ischemia when, exposed to locally released oxidants, platelet TXAZ/PGHZ receptors would be acutely increased. Under more normal conditions, receptor number could return to base line via enzymatic reduction. In this regard, it is interesting to note a recent report of patients with prolonged ischemia after myocardial infarction who acutely possessed a higher than normal density of platelet TXAZ/PGHZ receptors which returned to normal after convalescence (27).

In conclusion, the data presented herein provide evidence for disulfide and sulfhydryl group(s) at or near the ligand binding site of the human platelet TXAZ/PGHZ receptor. Oxidation rapidly activates, and reduction reversibly inactivates, the receptor. These findings suggest that heterospecific cyclic disulfide-sulfhydryl exchange reactions may acutely regulate human platelet TXAZ/PGHZ receptor number.

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Cyclic oxidation-reduction reactions regulate thromboxane A2/prostaglandin H2 receptor number and affinity in human platelet membranes.

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