Enhancement of the Binding of O-Ethyl O-p-Nitrophenyl Phenylphosphonate (EPNoxon) to Microsomal Carboxylesterase by NAD in vitro

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Accepted September 25, 1984

Abstract—Inhibition of rat liver microsomal carboxylesterase (CEase) by O-ethyl O-p-nitrophenyl phenylphosphonothioate (EPN) and binding of EPN oxygen analog to microsomal CEase were enhanced by addition of NAD or NADP. This was more prominent in addition of NAD than NADP. No potentiation of anti-CEase action of EPN by NAD was seen when pure esterase (E.C. 3.1.1.1) instead of liver microsomes was used as an enzyme source. This effect of NAD in microsomal CEase was significantly decreased when N-ethylmaleimide or p-chloromercuribenzoic acid was added. From these findings, it is strongly suggested that NAD-mediated potentiation of the anti-CEase action of EPN might be attributed to the increase in formation of NADH from NAD by microsomal dehydrogenase(s) containing a sulfhydryl group, leading to a subsequent increase in formation of the EPN oxygen analog from EPN, and in turn, CEase inhibition was enhanced.

Our previous papers revealed that addition of NAD to the incubation mixture containing rat liver microsomes and organophosphorothioates such as EPN and parathion can enhance the anti-carboxylesterase (CEase) action of these insecticides, but to a lesser extent in acetylcholinesterase (1). This phenomenon has been referred to as the “NAD-effect” in this study. Furthermore, the NAD-effect was not seen with the respective organophosphates and was found only when either β-NAD or 3-acetylpyridine adenine dinucleotide among five derivatives of NAD is added. This was closely related to the function of microsomal dehydrogenase(s).

The present study was undertaken to further investigate the characteristics of microsomal dehydrogenase(s) responsible for occurrence of the NAD-effect and the relationship between covalent binding of the EPN oxygen analog to microsomal CEase and the enzyme inhibition.

Materials and Methods

Animals: Adult male rats of the Wistar strain, weighing 200–230 g, were used. They were maintained on standard laboratory chow (Oriental Yeast Co., MF) and tap water ad libitum under the condition of constant room temperature (23–25°C) for at least one week before use.

Preparation of liver microsomes: The rats were sacrificed by decapitation, and the livers were immediately removed. Perfusion with 1.15% KCl was carried out to remove the blood after weighing the liver. Then, two volumes of ice-cold 1.15% KCl was added to give a 33% (w/v) homogenate. The homogenate was centrifuged at 9,000×g for 20 min, followed by recentrifugation of the
supernatant at 105,000×g for 60 min. The resulting pellets were washed once with ice-cold 1.15% KCl.

**Enzyme assay:** CEase activity was determined using the colorimetric assay procedure based on conversion of isocarboxazid to benzylhydrazine (BZH) as described previously (1, 2).

**Determination of binding of labelled-EPN to liver microsomal protein:** After incubation of phenyl-14C-EPN with NAD(P) and liver microsomes for 10 min, reaction was terminated by the addition of 15% metaphosphoric acid solution, and the precipitated microsomal proteins were separated by centrifugation at 3,000 rpm for 10 min. The supernatant fraction was removed and the pellet was washed vigorously with 15% metaphosphoric acid solution. Then, methanol was added to wash the pellet several times until no further radioactivity was detected in the supernatant. The final pellet was redissolved in 1N sodium hydroxide solution at 80°C. The 14C-radioactivity was measured by adding 10 ml aliquots of AQUASOL and counted by using a liquid scintillation counter (Beckman, model LS-100C), correcting for background and quenching.

The protein concentration of the liver microsomes was determined according to the method of Lowry et al. (3) using crystalline bovine serum albumin as a standard.

**Results**

**Effects of NAD on EPN-induced inhibition of rat liver microsomal CEase and pure esterase:** In order to determine the requirement of microsomal factor(s) responsible for potentiation of EPN-induced inhibition of CEase by NAD (NAD-effect), microsomal CEase was compared to that with pure esterase (E.C. 3.1.1.1). As shown in Table 1, no NAD-effect was seen when pure esterase was used.

**Effects of NAD and NADP on the EPN-induced inhibition of CEase and the binding of 14C-EPN to microsomal protein:** As shown in Fig. 1, EPN-induced inhibition of microsomal CEase was potentiated by addition of either NAD and NADP. A sigmoidal curve was seen in the CEase activity as a function of NAD concentrations ranging from 1×10⁻⁶ M to 1×10⁻⁴ M. On the other hand, the binding of 14C-radioactivity to microsomal protein was increased in parallel with concentrations of NAD, and a lesser extent of the NAD-effect was observed by addition of NADP instead of NAD.

**Effect of sulfhydryl reagents on NAD-effect:** As shown in Table 2, no NAD-effect was seen in presence of N-ethylmaleimide (NEM) (1 mM) or p-chloromercuribenzoic acid (PCMB) (0.1 mM), but not when iodoacetic acid (IAA) (1 mM) was added.

**Effects of pretreatment of liver microsomes with EPN and sulfhydryl reagents on NAD-effect:** Three inhibitors were used to clarify the possibility that sulfhydryl group(s) in liver microsomes might be involved in the NAD-effect. The incubation mixture consisting of 3 mg protein of microsomal pellet, 1 mM of EPN and 3 mM of sulfhydryl reagents was preincubated at 37°C for 10 min. Then, dialysis was done to remove surplus EPN and sulfhydryl reagents at 4°C for 24 hr in 1.15% KCl. Pure esterase (E.C. 3.1.1.1), 0.125 U, was added to the incubation mixture at 37°C for 60 min. The resulting pellets were washed once with ice-cold 1.15% KCl.

<table>
<thead>
<tr>
<th>Addition</th>
<th>CEase activity</th>
<th>Pure esterase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>65.7±2.8</td>
<td>122.1±6.3</td>
</tr>
<tr>
<td>EPN</td>
<td>61.0±3.3</td>
<td>100.1±4.1</td>
</tr>
<tr>
<td>EPN+NAD</td>
<td>9.2±4.6*</td>
<td>101.0±4.6</td>
</tr>
</tbody>
</table>

Activities of microsomal carboxylesterase (CEase) and pure esterase (from hog liver microsomes, E.C. 3.1.1.1) were shown as nmoles BZH formed/mg protein/30 min. The concentrations of EPN and NAD were 1×10⁻⁶ M and 5×10⁻⁶ M, respectively. Each value is the mean±S.E. from three experiments. *P<0.01: statistically significant difference from the EPN addition group.
Fig. 1. Effects of NAD and NADP on the EPN-induced inhibition of carboxylesterase and binding of $^{14}$C-EPN to microsomal protein. Inhibition percent of carboxylesterase activity and amount of $^{14}$C-EPN bound to microsomal protein (pmoles $^{14}$C-bound/mg protein/10 min) are shown in A and B of the figure. The concentration of EPN used was $1 \times 10^{-5}$ M. Each point is the mean of three experiments. —○—: NAD, —●—: NADP.

Table 2. Effects of sulfhydryl reagents on the NAD-mediated potentiation of anti-carboxylesterase action of EPN in vitro

<table>
<thead>
<tr>
<th>Addition</th>
<th>None</th>
<th>NEM 0.1 mM</th>
<th>NEM 1 mM</th>
<th>PCMB 0.01 mM</th>
<th>PCMB 0.1 mM</th>
<th>IAA 0.1 mM</th>
<th>IAA 1 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.0±3.5</td>
<td>53.2±3.3</td>
<td>40.6±1.7</td>
<td>57.4±5.0</td>
<td>52.7±4.7</td>
<td>54.6±2.7</td>
<td>51.3±3.5</td>
</tr>
<tr>
<td>EPN</td>
<td>52.2±3.4</td>
<td>49.1±3.2</td>
<td>38.6±1.4</td>
<td>52.3±4.7</td>
<td>47.9±4.7</td>
<td>49.8±3.0</td>
<td>46.5±2.8</td>
</tr>
<tr>
<td>EPN+NAD</td>
<td>3.5±0.5*</td>
<td>3.8±0.4*</td>
<td>34.2±1.7</td>
<td>6.9±2.7*</td>
<td>45.6±5.4</td>
<td>3.3±0.5*</td>
<td>3.3±0.6*</td>
</tr>
</tbody>
</table>

Carboxylesterase activity was shown as nmoles BZH formed/mg protein/30 min. The concentrations of EPN and NAD used were $1 \times 10^{-5}$ M and $5 \times 10^{-5}$ M, respectively. Each value is the mean±S.E. from 3–5 experiments. NEM: N-ethylmaleimide, PCMB: p-chloromercuribenzoic acid, IAA: iodoacetic acid. *P<0.01: statistically significant difference from the EPN alone group.
mixture for CEase assay in this study. As shown in Table 3, the NAD-effect still remained when IAA-pretreated microsomes were used, but not with NEM- or PCMB-pretreatment.

Effects of sulfhydryl reagents on the NAD-effect and binding of 14C-EPN to microsomal protein: Based on the fact that the NAD-effect was not seen by addition of NEM or PCMB, we investigated the effect of 1 mM of sulfhydryl reagents on the binding of 14C-EPN to microsomal protein. As shown in Fig. 2, the potentiation of EPN-induced inhibition of CEase by NAD and the increase in binding of 14C-radioactivity to microsomal protein by NAD were observed in the absence of sulfhydryl reagents. On the other hand, addition of NEM or PCMB to the incubation mixture resulted in no difference in the binding of 14C-radioactivity to microsomal protein between in the presence or absence of NAD. This result showed a good correlation with the effects of NEM or PCMB on CEase activity. In the case of IAA, however, a significant inhibition of CEase activity and increase in the binding of 14C-radioactivity to microsomal protein were observed.

Discussion

In the previous papers, we reported that addition of NAD to the incubation mixture containing rat liver microsomes and EPN enhanced inhibition of microsomal CEase by EPN (1). In addition, the fact that no NAD-effect was observed when pure mixture for CEase assay in this study. As shown in Table 3, the NAD-effect still remained when IAA-pretreated microsomes were used, but not with NEM- or PCMB-pretreatment.

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Discussion

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esterase (E.C. 3.1.1.1) instead of liver microsomes was used, suggested that unknown factor(s), presumably dehydrogenase(s), present in liver microsomes was required to show a potentiation of the anti-CEase action of EPN by NAD.

The present paper revealed that binding of $^{14}$C-EPN to liver microsomal protein was also markedly increased when NAD was added to the incubation mixture. Further, potentiation of the anti-CEase action of EPN and increase of binding of $^{14}$C-radioactivity to microsomal protein by NAD were both blocked by addition of sulfhydryl reagents. Therefore, it is likely that EPN added might be oxidized to EPNoxon by liver microsomal dehydrogenase(s) possessing sulfhydryl groups which require mainly NADH as a cofactor; then the EPNoxon formed was bound to liver microsomal CEase. Our preliminary study revealed that the enzymatic formation of EPNoxon was seen when NAD was added to the incubation mixture for CEase assay (Table 4). Unlike many other foreign compounds, several papers report that NADH is 40-80 percent as effective as NADPH in the oxidative reaction by cytochrome P-450-coupled monooxygenase with NADH which was formed from NAD by microsomal membrane-bound dehydrogenase(s). Further studies are now in progress.

Acknowledgment: We are grateful to Miss. Mari Takamiya for excellent technical assistance.

References
6 Shishido, T., Usui, K. and Fukami, J.: Oxidative

### Table 4. Effects of NAD on the enzymatic formation of EPNoxon from EPN and carboxylesterase activity

<table>
<thead>
<tr>
<th>Addition</th>
<th>EPNoxon (M)</th>
<th>% (EPNoxon/EPN)</th>
<th>Carboxylesterase (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>—</td>
<td>100.0</td>
</tr>
<tr>
<td>EPN</td>
<td>N.D.</td>
<td>—</td>
<td>86.9</td>
</tr>
<tr>
<td>EPN+NAD</td>
<td>$3.5 \times 10^{-7}$</td>
<td>2.3</td>
<td>13.3</td>
</tr>
<tr>
<td>EPN+NAD+PMSF+EDTA</td>
<td>$4.7 \times 10^{-7}$</td>
<td>3.1</td>
<td>—</td>
</tr>
</tbody>
</table>

The concentrations of EPN added were $1.5 \times 10^{-6}$ M in 10 ml of incubation mixture for the EPNoxon assay and $1 \times 10^{-6}$ M in 1.5 ml of incubation mixture for the carboxylesterase assay, respectively. The concentrations of NAD used were $7.5 \times 10^{-6}$ M for the EPNoxon assay and $5 \times 10^{-6}$ M for the carboxylesterase assay, respectively. PMSF and EDTA added were 1 mM and $4.5 \times 10^{-8}$ M, respectively. N.D.; less than $0.5 \times 10^{-7}$ M. Each value is the mean of two experiments. EPNoxon was determined by the GC-MS method.


