Determination of Reduced Nicotinamide Adenine Dinucleotide Phosphate Concentration Using High-Performance Liquid Chromatography with Fluorescence Detection: Ratio of the Reduced Form as a Biomarker of Oxidative Stress

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Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is the principal source of reducing power in numerous processes of physiological importance. We examined the influence of oxidative stress on the relative amounts of NADPH in human red blood cells (RBCs). To determine the homeostasis of the NADPH existing in the reduced form following oxidation, we developed an improved method for measurement of NADPH in human RBCs using high-performance liquid chromatography (HPLC). The present method with fluorescent detection is reproducible and selective than enzymatic cycling method and HPLC methods with spectrometric detection. The calibration curve is linear over the range of 0.1—5.0 μmol with a correlation coefficient of 0.999. The within-run precision of the assays for total NADPH (NADPH+NADP⁺) and NADPH concentrations in human RBC were 2.4% and 8.6%, respectively (n=5). After the RBC suspension was exposed to tert-butyl hydroperoxide (t-BHP), which is scavenged by glutathione peroxidase (GPX) along with NADPH consumption, a significant decrease in the NADPH ratio [(NADPH/(NADPH+NADP⁺)] was observed after a transient decrease and rapid recovery of the reduced form of glutathione. The marked decrease in the NADPH ratio induced by t-BHP exposure was observed in the absence of glucose. However, the NADPH ratio was not decreased by t-BHP exposure after pretreatment with a glutathione reductase inhibitor. This method may be useful for the measurement of small amounts of NADPH from various biological sources.

Key words  NADPH ratio; oxidative stress; red blood cell; reactive oxygen species; tert-butyl hydroperoxide

Reduced nicotinamide adenine dinucleotide phosphate (NADPH), the principal intracellular reductant, plays a key role in providing reducing equivalents in living cells to protect against oxidative damage.1) The ratio of reduced to oxidized forms [NADPH]/[NADP⁺] is approximately 10 : 1 in most cell types.2) By acting as the electron donor cofactor in the glutathione (GSH) reductase (GR) reaction, NADPH is critical in maintaining appropriate levels of GSH, the most abundant source of non-protein thiol.s. A number of investigations have suggested that the [GSH]/[oxidized glutathione (GSSG)] ratios is 50—100 in the cytosol of mammalian cells.3) Thus, intracellular levels of NADPH seem to be controlled to maintain the cell redox potential in a manner dependent upon GSH. However, it is less clear how regulation of the NADPH ratio [(NADPH/(NADPH+NADP⁺)] changes with oxidative damage, and this is a crucial point for understanding the mechanisms controlling redox homeostasis in mammalian cells.

NADPH is generated by a variety of reactions. It is known that mammalian tissues contain three NADP⁺-linked dehydrogenases: glucose 6-phosphate dehydrogenase (EC: 1.1.1.49), isocitrate dehydrogenase (EC: 1.1.1.42), and malic enzyme (EC: 1.1.1.40).4) However, it is generally believed that in many cells, including red blood cells (RBCs) and hepatocytes, a major part of the NADPH requirement is met by oxidation of the glucose 6-phosphate pathway (PPP).5) Furthermore, the role of glucose phosphate dehydrogenase (G6PDH) in cellular response to oxidative stress is well established in human RBCs.6) Conversely, RBCs have been used as a model in numerous studies to determine the significance of the GSH-dependent redox system since RBCs have an efficient antioxidant defense system composed of enzymes that function to prevent oxidative damage.7—9)

It is apparent that the maintenance of the NADPH ratio is required in redox systems; however, to the best of our knowledge there are few reports describing in detail the change in the NADPH ratio induced by reactive oxygen species (ROS). Several methods exist to quantify pyridine nucleotides, both the oxidized form (NADP⁺) and the reduced form (NADPH).10—18) The method developed by Zerez et al.11) offered several advantages over earlier methods. However, while using this method, we observed that the experimental system was incapable of quantifying NADPH that is protein bound, thus changing the ratio of reduced NADPH to total NADPH (NADPH⁺+NADPH). Next, we adapted the modified method by Wagner and Scott12) to more reliably measure NADPH in human RBCs. Unfortunately, we obtained the unexpected result that the NADPH ratio was greater than the theoretical maximum of 1.0. We were also troubled by the low reproducibility of enzymatic cycling assay and its complicated reagents preparation and procedures, which lack an automated system. Thus, we established an improved method for selectively determining NADPH and total NADPH (NADPH⁺+NADPH). The present method utilizes HPLC and allows us to assess changes in the NADPH ratio in human RBCs in order to study redox regulation during exposure to tert-butyl hydroperoxide (t-BHP). Our study clearly shows that the NADPH ratio is changed in response to ROS exposure in RBCs and that the ratio depends upon glucose levels regulating PPP. Moreover, the data indicate that, when RBCs are exposed to ROS primary consumption of NADPH occurs during the reduction of GSSG to GSH.
MATERIALS AND METHODS

**Chemicals** NADPH, NADP⁺, nicotinamide, GSH, t-BHP, glucose 6-phosphate, G6PDH, and 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU) were purchased from Sigma (St. Louis, MO, U.S.A.). Dithiothreitol, ADP, ATP, and glucose assay kits were obtained from Wako Pure Chemical Co. (Osaka, Japan). Bovine serum albumin (BSA) was obtained from Boehringer-Mannheim Co. (Darmstadt, Germany). HPLC grade methanol was purchased from Merck Co. (Darmstadt, Germany).

**Preparation of Human Red Blood Cell Suspensions** Twelve-hour fasting blood samples were obtained from seven healthy subjects (aged 21—45) after they gave informed consent. Blood was drawn into vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA). RBCs were centrifuged for 15 min at 4 °C, 800 g and were washed twice in phosphate buffered saline (PBS) with the same centrifugation procedures. The RBC suspension was prepared by adding PBS in the presence or absence of 10 mM glucose. The hematocrit of each RBC suspension was adjusted to 40% (Hct. 0.40). All experiments were performed on the day blood was drawn.

**Development of NADPH Ratio Assay** Total NADPH (NADPH + NADP⁺) concentrations were determined by fluorometric detection with HPLC using 50 μl of washed and packed RBCs that were lysed by the addition of 950 μl of a 100 mM nicotinamide solution. Two hundred microliters of the sample lysate was added to 130 μl of 0.165 M Tris/HCl (pH 8.0) containing 16.5 mM MgCl₂, 8.3 mM G6P, and 8.3 U/ml G6PDH. In the case of NADPH determination, the same amount of the sample lysate was added to 130 μl of 0.165 M Tris/HCl (pH 8.0) in the absence of G6P and G6PDH. After incubation at 37 °C for 5 min, 0.5 ml of 0.12 mM carbonate buffer (pH 12.0) was added to the mixture. Next, the solution (pH 10.4) was incubated at 60 °C for 30 min, followed by centrifugation at 4 °C (15000 g for 5 min). The reaction mixture was ultrafiltered (cutoff size: 10000, Millipore, Japan) at 4 °C, after which 100 μl of the filtered solution was submitted to an HPLC system consisting of a reverse phase column (4.0×250 mm, LiChrosphere RP-18(e), 5 μm, Merck), which had been pre-equilibrated with a mobile phase comprised of 5% methanol and 95% 0.1 M phosphate buffer at pH 6.0. A standard curve was produced from stock solutions of NADPH in extraction buffer (0.12 mM carbonate buffer, pH 12.0, containing 10 mM nicotinamide) over a range of 10—500 μM (final concentration: 0.1—5.0 μM NADPH). A flow rate of 1.0 ml/min was used with a running time of 15 min.

Retention times and peak areas were monitored at excitation and emission wavelengths of 340 nm and 460 nm, respectively. NADPH concentrations were extrapolated from the areas underneath given calibration curves.

Total NADPH was determined by enzymatic treatment using G6P and G6PDH to convert NADP⁺ to NADPH. NADPH concentrations within RBCs were determined using a similar procedure, except for the enzymatic conversion step. The ratio of NADPH was calculated by dividing the amount of NADPH detected by the total amount of NADPH (NADPH + NADP⁺).

**Recovery Test** Analytical recovery was performed by adding a known amount of NADP⁺ or NADPH at 5 μM in 0.1 M phosphate buffer (pH 6.5) or a blank solution (only vehicle) to a pooled RBC suspension. The samples were treated as described above.

**Oxidation of Human RBC Suspensions Exposed to t-BHP** The effects of t-BHP were examined in RBC suspensions in the presence or absence of 10 mM glucose by incubating the RBCs at 37 °C with 2.0 mM t-BHP for 60 min, after which the RBC suspensions were centrifuged for 10 min at 4 °C, 800 g and washed twice to remove extracellular t-BHP. The packed RBCs was applied to determine the GSH levels and NADPH concentrations.

**GSH Determination** RBCs in suspension were centrifuged, and the packed cells were lysed by the addition of eight volumes of distilled water and the immediate addition of one volume of 25% (w/v) sulfosalicylic acid. After centrifugation for 5 min at 15000 g, a clear supernatant was obtained and then used for determination of GSH. GSH was measured by an HPLC method previously reported with minor modifications. Briefly, the clear supernatant (10 μl) of acid-treated samples was neutralized by addition of four volumes of 0.2 M borate buffer (pH 10.5), and the mixture (50 μl) was allowed to react with labeling reagent. The concentration of GSH in RBCs was calculated by multiplying the value in the lysate by ten.

**Other Methods** The total protein concentration was determined by the Lowry method using BSA as a standard (Bio Rad, Assay Kit). RBC glucose content was determined by colorimetric assay using glucose oxidase (Wako, Assay kit).

**Statistics** Each experiment was performed at least four times, and the results are expressed as means±S.D. Data were compared using Student’s t-test. Statistical significance was considered at p<0.05.

RESULTS

**Determination of NADPH and Total NADPH (NADPH + NADP⁺) in Normal Human RBCs** To study changes in oxidative status, we devised a practical method to determine the NADPH concentration in RBCs as a model of living cells. We adopted a modified HPLC method with fluorometric detection using a general reverse phase column. This technique is relatively simple to perform and yields sensitive results over 5 times compared to the spectrometric assay methods. An isocratic solvent system allowed for sufficient separation of NADPH from RBCs on a C₁₈ col-
method agreed with those of previous experiments \textsuperscript{10,11} using \( \text{NADPH} \) signal-to-noise ratio of 3 per 100 \( \text{m} \). The minimum detectable level was 1 pmol of \( \text{NADPH} \) with a range of 0.1—5.0 \( \text{m} \).

Fig. 1. The calibration curve for \( \text{NADPH} \) was linear over the retention time for \( \text{NADPH} \) (6.3 min) is shown in Table 1, under optimal conditions, total \( \text{NADPH} \) was 2.4% and 8.6%, respectively. As shown in Table 1, under optimal conditions, total \( \text{NADPH} \) (\( \text{NADPH} + \text{NADP}^+ \)), \( \text{NADPH} \) and their ratio \( \text{[NADPH]} / (\text{NADPH} + \text{NADP}^+) \) in human RBCs obtained by our method agreed with those of previous experiments\textsuperscript{10,11} using spectrophotometric assays. The procedure for RBC lystate preparation allows for good recovery of \( \text{NADP}^+ \) and \( \text{NADPH} \) (\( n=3 \), over 93.0% and 95%, respectively).

Alterations in the NADPH Ratios and GSH Levels upon Exposure of the RBC Suspension to \( \text{t-BHP} \). In the presence of glucose, GSH levels in the RBC suspensions did not change (Fig. 2A), while reduced NADPH ratios declined significantly decreased following one-hour incubation with \( \text{t-BHP} \) (Fig. 2B).

Effect of Low Intracellular Glucose on the NADPH Ratio Following Exposure of the RBC Suspension to \( \text{t-BHP} \). After incubating the RBC suspensions for 45 min in the absence of glucose, the RBC suspension was exposed to \( \text{t-BHP} \) and incubated for one hour in the absence of glucose. As shown in Fig. 3, GSH levels and the NADPH ratio significantly decreased. The NADPH ratio and GSH level had not recovered 4 h after \( \text{t-BHP} \) exposure in the RBC suspension pre-incubated in glucose-free medium (data not shown).

Effect of BCNU Treatment on the NADPH Ratio Following Exposure of the RBC Suspension to \( \text{t-BHP} \). When GR is inhibited or the glucose concentration is low, reduction of GSSG and the regeneration of \( \text{NADPH} \) via PPP might not occur as shown in Fig. 4. We used BCNU, a specific inhibitor of GR,\textsuperscript{21} to inhibit the consumption of \( \text{NADPH} \) via the reduction of GR-dependent GSSG. After treatment with 1 mM BCNU and 10 mM glucose, the RBC suspension was exposed to \( \text{t-BHP} \) and incubated for 1 h in the presence of glucose. Figure 5 shows the observed change in GSH level (Fig. 5A) and NADPH ratio (Fig. 5B). A significant decrease in the NADPH ratio was not observed in BCNU-treated RBCs and untreated RBCs following exposure to \( \text{t-BHP} \). However, a significant difference in GSH levels was observed in BCNU-treated RBCs exposed to \( \text{t-BHP} \).

**DISCUSSION**

\( \text{NADPH} \) is an essential cofactor for the regeneration of GSH by GR, in addition to its critical role in the activities of the \( \text{NADPH-dependent glutaredoxin and thioredoxin} \) sys-
Fig. 4. Diagram of the Pentose Phosphate Pathway and NADPH Consumption

Scheme represents the metabolic pathways normally operating in human RBCs to maintain the reduced forms of NADP and GSH. Peroxides exposure induces the oxidation of GSH to form GSSG. The pentose phosphate pathway is accelerated to maintain the levels of NADPH and GSH. See Discussion for details.

Fig. 5. Effects of BCNU Pre-treatment on GSH Levels and NADPH Ratios in RBCs Exposed to t-BHP

Human RBC suspensions (Hct. 0.50) in PBS containing 10 mM glucose were incubated in the presence (BCNU) or absence (Control) of 1 mM BCNU for 30 min. After centrifugation to remove excess BCNU and re-suspension in PBS containing 10 mM glucose, each RBC suspension (Hct. 0.40) was incubated with 2 mM t-BHP at 37 °C for 60 min. (A) GSH concentrations within the RBCs were determined as described in Fig. 4. (B) After centrifugation at 4 °C and 800 g for 10 min, the packed RBCs were lysed by the addition of 19 volumes of cooled 100 mM nicotinamide solution. NADPH and total NADPH (NADPH+/NADP+) in the hemolysate samples (n=4) were assayed as described in Materials and Methods. **p<0.01.

Although both are important in protecting cells from oxidative damage, it is likely that NADPH primarily plays a role of supplying reducing potentials for the GSH-dependent redox system in RBCs.22—24) Scott et al. suggested that NADPH status modulates oxidant sensitivity in normal and G6PDH-deficient erythrocytes.6) Furthermore, Cheng et al. demonstrated that both G6PDH activity and the NADPH/NADP+ ratio progressively decline as the amount of 8-hydroxy deoxyguanosine level increases during serial propagation of G6PDH-deficient cells.25) Thus, we focused on the decrease in the NADPH ratio [NADPH/(NADP++NADPH)] as a significant marker for early detection of changes in redox balance.

We established an improved method to precisely quantify NADPH and total NADPH in RBCs. Although some reports have described the HPLC method for spectrometric detection of nucleotides including NADPH as being time-consuming and generally less sensitive and specific than enzymatic cycling methods,23,24) the HPLC method presented here is more sensitive and specific for NADPH than usual HPLC methods combined with spectrometric detection due to the use of fluorometric detection. In addition, our method is practical and convenient for multiple samples since it is compatible with an auto-sampler. Our method has advantages over other methods for measuring alterations in the NADPH ratio.12,15—17) It appears that other investigators observed lower NADPH ratios than the true values2,22) because the NADPH ratio is constitutively maintained nearly 1.0 in living cell on normal condition.5,25) The reason that the lower NADPH ratio was measured in normal RBCs or living cells, despite following an alkaline extraction procedure, is likely because these studies omit the heating step intended to release NADPH from binding protein13,29,30) and both NADP and NADPH are determined simultaneously. Indeed, Lowry et al. reported that during alkaline extracts NADPH is oxidized and subsequently destroyed if hemoglobin is present.12,31,32) Thus, we chose to perform hemolysis of packed RBCs under neutral conditions, and complete lysis was achieved by the addition of a 20 fold of low isotonic solution containing nicotinamide. In addition, we found that rapid addition of G6PDH and G6P to RBC lysate in Tris/HCl buffer (pH 8.0) was useful for recovering spiked NADP+. Incubation for 30 min at 60 °C is essential for the sufficient recovery of NADPH from RBC suspensions because alkaline extraction buffer does not denature/precipitate known NAD(P)H binding proteins in RBCs, e.g., catalase.29,30) Although the HPLC method for detecting NADPH in cultured rat hepatocytes by monitoring the fluorescence of NADPH has previously been reported,33) NADPH and NADP+ were independently determined using two extraction procedures and two corresponding detectors. Another HPLC method has been proposed for analysis of oxidized and reduced pyridine nucleotides in rodent brain.34) The specific method is highly sensitive with simple extraction procedures and allows for tracing NADP+ and NADPH on one chromatogram. However, this unique method requires cyanide and leads to two fluorescent products generated from the oxidized form (NADP+). Additionally, a gradient elution requires over 30 min for each assay and methemoglobin is highly reactive to cyanide. Therefore, this method is time-consuming and inadequate for NADPH analysis in RBCs.

A disadvantage of our method presented here is the necessity to divide the sample into two aliquots for analysis of the reduced form (NADPH) and total NADPH (NADP++NADPH). However, present method allows us to obtain better chromatographic separation of NADPH (Fig. 1) using a simple eluent under isocratic conditions within 15 min. Furthermore, since the solution is alkalized (pH 10.4) after enzymatic conversion to NADPH, the matrix is suitable for NADPH stability, and an autosampler can be used.

In this study, we initially used t-BHP and hydrogen peroxide as sources of ROS. However, no significant differences were observed in the NADPH ratios when hydrogen peroxide was added to RBCs because of the rapid scavenging by catalase.35) Thus, we measured changes in the NADPH ratio during exposure to t-BHP under different conditions of oxidative stress in association with a GR inhibitor or glucose deficiency.

Figure 2 indicates that the NADPH ratio was affected by peroxide exposure and that the ratio did not recover immediately in human RBCs in vitro. We previously demonstrated that GSH and GSSG levels in intact RBCs transiently decreased and increased upon exposure to t-BHP, respectively but rapidly recovered within 1 h.36) These observations suggest that t-BHP is scavenged via a GPX-catalyzed reaction with GSH (Fig. 4). Additionally, a decrease in GSH levels...
and an increase in GSSG levels were clearly observed in RBCs exposed to t-BHP when the RBCs were pre-treated with 1 mM BCNU or pre-incubated in glucose-free medium. In the current study, a clear decrease in the NADPH ratio was observed following exposure to t-BHP under low levels of glucose in RBCs, and the recovery of the ratio was significantly impaired (Fig. 3). These findings indicate that regeneration of NADPH is dependent upon reactions with G6PDH and 6-phosphogluconate dehydrogenase via PPP, as shown in Fig. 4. However, it is also well known that exposure to t-BHP induces the formation of methemoglobin. Consequently, glucose in t-BHP-exposed RBCs metabolize via glycolysis in order to maintain a balance of NADH, which is a required cofactor in the reduction of methemoglobin by methemoglobin reductase. The acceleration of glycolysis results in a decrease in the NADPH ratio may be a useful biomarker for imbalance in the pentose phosphate pathway but it also indicates that the maintenance of GSH levels in human RBCs. Moreover, a decrease in the ratio might serve as a biomarker for imbalanced redox regulation in the cells. In addition, the proposed methodology described in this article allows for simple and accurate determination of NADPH levels in individual RBCs or clinical samples, and it should aid in elucidating the role of NADPH in the cellular redox balance. The NADPH ratio is affected when NADPH-dependent reductases act to recover the normal redox balance, and regulation of the ratio depends on glucose metabolism. Therefore, NADPH ratios are altered along with NADPH consumption primarily due to the maintenance of GSH levels in human RBCs. Moreover, a decrease in the ratio might serve as a biomarker for imbalanced redox regulation in the cells. In addition, the proposed method may be useful for studying oxidative stress because changes in the NADPH ratio would be maintained for a long periods of time compared to changes in GSSG levels. This in vitro study suggests not only that NADPH contributes to the glutathione-dependent antioxidant system, which is dependent upon the pentose phosphate pathway but it also indicates that a decrease in the NADPH ratio may be a useful biomarker for exposure to reactive oxygen species and dysfunction of the redox system in living cells.

REFERENCES