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Mass Spectral Analyses of Hydroxymethylvinyl Ketone-Hemoglobin Adducts Formed after in Vivo Exposure of Sprague-Dawley Rats to 3-Butene-1,2-Diol

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Abstract

3-Butene-1,2-diol (BDD), a known in vivo metabolite of 1,3-butadiene, is oxidized to a reactive Michael acceptor, hydroxymethylvinyl ketone (HMKV). Previously, we characterized formation of three HMKV-amino acid monoadducts when HMKV was incubated in vitro with N-acetyl-L-cysteine (NAC), L-valinamide, and N-acetyl-L-lysine (NAL) at physiological conditions. One HMKV-NAL cyclic diadduct (cyclic diadduct 1) also formed by sequential Michael addition reactions of two HMKV molecules with the ϵ -amino group of NAL followed by enolization and cyclization. Loss of a water molecule and autooxidation converts cyclic diadduct 1 to a more stable cyclic diadduct 2. In the present study, we used multiple mass spectrometry techniques to investigate formation of HMKV adducts with nucleophilic residues of Hb in vivo after dosing Sprague-Dawley (SD) rats with 25 and 200 mg/kg BDD. Trypsin digested globin peptides with mass shifts consistent with the presence of HMKV monoadducts and cyclic diadducts were detected by LC/ESI-QTOF/MS with all rats given BDD. Use of MALDI/FTICR provided further evidence for formation of HMKV monoadducts and cyclic diadducts and use of LC/MS/MS provided unequivocal evidence for adduction of HMKV with Cys125 of globin β chains. Because BDD can also be oxidized to 1,2-dihydroxy-3,4-epoxybutane (EBD), formation of N²-(2,3,4-trihydroxybutyl) (THB)-Hb adducts was also investigated in rats given BDD and several peptides modified by THB were detected. However, because HMKV incubations with red blood cells in vitro also led to detection of THB-Hb adducts, the THB adducts formed in vivo could be attributed to formation of HMKV, EBD, or both. Collectively, the results provide new insights into the reaction of HMKV with proteins.

Introduction

3-Butene-1,2-diol (BDD) is a known metabolite of 1,3-butadiene (BD) (1,2,3), an occupational and environmental chemical that is classified by the International Agency for Research on Cancer as “carcinogenic to humans” (4). In addition to causing multi-organ tumor formation in rodents (5,6), long-term BD exposure has also been associated with anemia, bone marrow toxicity, and testicular and ovarian atrophy (5). When BDD or its precursor, 1,2-epoxy-3-butene (BMO) (Figure 1), were administered to rodents, only a small fraction (<5%) of the dose was detected in the urine as BDD, suggesting extensive further metabolism of BDD (7, 8).

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Supporting Information Available Tables that contain intact globin data modified by HMKV, cyclic diadduct 2, and THB for in vivo samples from rats given BDD are available in the supplemental section. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Our laboratory had previously shown that BDD can be oxidized by cytochrome P450s or alcohol dehydrogenases to yield hydroxymethylvinyl ketone (HMK) (9,10,11) (Figure 1), a reactive Michael acceptor that readily reacts in vitro with nucleophiles such as GSH (11) and biological macromolecules, such as Hb (12). However, following mouse or rat treatment with BDD, only 4-8% of the dose was recovered in urine as HMK-derived mercapturates suggesting that a large fraction of the BDD dose may be HMK bound to macromolecules (13).

We have previously investigated formation of HMK adducts with nucleophilic amino acids that serve as models for nucleophilic residues of Hb and characterized HMK monoadducts resulting from Michael addition reactions of N-acetyl-L-cysteine (NAC), N-acetyl-L-lysine (NAL), and L-valinamide with HMK (14) (Figure 2). We have also characterized two cyclic HMK-NAL diadducts consisting of two HMK moieties that formed as a result of a bis-Michael addition reaction (14) (Figure 2). We proposed that cyclic diadduct 1 formed via enolization of one of the HMK moieties followed by cyclization to form an octameric ring which further undergoes loss of water and autooxidation resulting in cyclic diadduct 2 (Figure 2). We have further used MS techniques to characterize HMK-Hb monoadduct formation in vitro when we incubated 0.1 and 1 mM HMK with red blood cells (RBCs) at physiological conditions (12). Our data suggested that HMK monoadducted Cys125-containing peptides on globin β chains (105-132, 121-132, or 121-144) were present at both the high and low HMK concentrations providing evidence for selectivity of these peptides in the reaction of HMK with Hb. Because adducts with more than one HMK moiety on these peptides were detected (12), the results suggested that nucleophilic residues besides cysteines may also be involved in HMK reactivity consistent with the results with the model nucleophilic amino acids (14).

Formation of HMK-Hb adducts in vivo has not been characterized before unlike the N²-(2,3,4-trihydroxybutyl)valine (THB-Val) adducts (Figure 1) formed between 1,2-dihydroxy-3,4-epoxybutane (EBD) and N-terminal valines (15). EBD is a reactive metabolite of BD that could be formed by oxidation of BDD, hydrolysis of 1,2:3,4-diepoxybutene (DEB), or both (16,17,18). Currently, THB-Val adducts are used for human BD exposure monitoring (19). However, when rats and mice were exposed to 1,3-[2,3-¹⁴C]-butadiene, only 1-2% of the total globin radioactivity was due to THB adducts (20), suggesting reactive metabolites other than EBD may also be bound to globin. The relative contributions of bioactivation pathways of BDD leading to HMK or EBD formation are presently unknown.

In the present study, we used various MS techniques to investigate in vivo HMK and EBD formation after dosing of rats with BDD. Based on our previous characterization of monoadducts with NAC, NAL, and valinamide, we have analyzed for HMK monoadduct formation with sulfhydryl and amino groups of trypsin digested globin peptides (Figure 2). We have also analyzed for peptides modified by cyclic diadducts 1 and 2 with amino groups of Hb (Figure 2) based on characterization of similar cyclic diadducts by the reaction of HMK with NAL (14). Furthermore, we characterized formation of THB-Hb adducts after rat treatments with BDD to provide insights into possible EBD formation. In vitro incubations of RBCs with HMK were also carried out to obtain evidence for or against the involvement of HMK in formation of Hb cyclic diadducts and THB adducts.

Experimental Procedures

Caution

BDD and HMK are hazardous and should be handled with care.

Materials

Butyne-1,4-diol, boron trifluoride diethyl etherate, acetone, trifluoroacetic acid and deuterium oxide were purchased from Sigma-Aldrich Research (St. Louis, MO). Mercury (II) oxide and trichloroacetic acid were purchased from Fisher Scientific (Pittsburgh, PA). Trypsin (reductively alkylated) was obtained from Promega (Madison, WI). HMVK was synthesized as previously described (11) based on a modified Meyer-Schuster rearrangement method (21). Identity and purity were confirmed by GC/MS (Hewlett-Packard; Palo Alto, CA) (3) and ^1H NMR on Varian (Palo Alto, CA) Unity INOVA (400 MHz). Purity of at least 96% was achieved consistently.

Animals

Male Sprague-Dawley (SD) rats (210-285g) were purchased from Sasco (Omaha, NE). Rats were maintained on a 12 h light/dark cycle and given water and feed ad libitum. Rats were housed individually in metabolic cages. Three rats were each injected i.p. with a single 1 mL low dose (25 mg/kg BDD) at a concentration of 6.1 mg/mL saline. Two rats were injected with 1 mL of a higher BDD dose (200 mg/kg) at a concentration of 48.0 mg/mL saline. Both doses have been associated with the formation of HMVK in vivo as evidenced by presence of HMVK-derived mercapturic acids in rat urine (13). One rat was injected with saline to serve as a control. Rats were sacrificed 24 h after dosing by CO_2 asphyxiation. Heparinized whole blood was retrieved through cardiac puncture and globin isolated using acidified acetone as previously described (22,23). In vitro experiments using HMVK were also carried out as described previously (12). Briefly, freshly isolated erythrocytes from SD rats were incubated with 0.1, or 1 mM HMVK dissolved in 10 mM PBS (8.4 mM Na_2HPO_4 , 1.6 mM KH_2PO_4 , 154 mM NaCl), or only PBS for 2 h at physiological conditions (pH 7.4, 37°C) before globin isolation and analyses.

Mass Spectra of Tryptic Digest

Trypsin digestion of globin from control RBCs and RBCs exposed to BDD in vivo and HMVK in vitro was performed (22). Digest samples were dried and reconstituted in 50% acetonitrile (ACN):50% double deionized water (ddH_2O) before being subjected to Electrospray Hybrid Quadrupole Orthogonal Time-Of-Flight mass spectrometer (ESI-QTOF/MS; Waters Corporation, Milford, MA) equipped with a Zorbax C18 stable bond column (100 $\mu \times 17$ cm, 5 μm , 300 Å pore size; Agilent Technologies, Wilmington, DE). Solvent A consisted of ddH_2O , 0.1% formic acid and solvent B was ACN, 0.1% formic acid. The column was equilibrated at 98% Solvent A for 25 min, then %A was decreased to 90 over 75 min, then to 40 over 5 min before being taken down to 0 over 15 min and back up to 98% over 60 min at 30 $\mu\text{L}/\text{min}$. Spectra were scanned with a mass range between 50 to 2200 m/z . Multiply charged ion peaks were deconvoluted to generate a peak list of monoisotopic masses for each 10-25 min elution increment using MaxEnt function of MassLynx 4.0.

Trypsin digested peptides from one of the rats treated with 200 mg/kg BDD were also fraction collected using previously developed HPLC method (22) before further MS and tandem MS analyses in order to localize specific sites of peptide modification by HMVK. Dried and reconstituted fractions (in 20 μL methanol: 20 μL H_2O) were subjected to Varian IonSpec ProMatrix Assisted Laser-Desorption Ionization/Fourier Transform Ion Cyclotron Resonance (ProMALDI/FTICR) mass spectrometer (22).

Data Analyses of Trypsin Digest

The most abundant SD rat globin chains (labeled as $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$) with known molecular masses and sequences (www.ncbi.nlm.nih.gov) (24) were used in the analyses. Composition of $\alpha 1$ and $\alpha 2$ chains is 70 and 30%, respectively, and $\beta 1$ (also described as $\beta 3$ by Ferranti et. al)

and $\beta 2$ chains comprise 34.8 and 27.3 % of relative abundance of the β chains (24). A virtual list of trypsin digested peptides of SD rat Hb chains was generated using MS Digest tool in Protein Prospector (www.prospector.ucsf.edu). Monoisotopic masses for modified peptides (including one and two missed cleavages and up to 4 adducts) were then calculated for HMVK monoadducts (+86.0 Da for LC/ESI/MS and +86.0368 Da for MALDI/FTICR) and EBD-derived THB monoadducts (+104.0 Da for LC/ESI/MS and +104.0473 Da for MALDI/FTICR). Theoretical mass lists were also compiled for HMVK cyclic diadduct 2 (+152 Da for LC/ESI/MS and +152.0473 Da for MALDI/FTICR) and peptides analyzed for the presence of up to 4 adducts. Data tables containing deconvoluted monoisotopic experimental masses were imported into Microsoft Access where specific queries were set up to search for mass matches. The criteria for finding modified peptides were as follows: the peak representing a monoisotopic mass that was a match was absent in control samples and allowable mass error was set to ± 0.5 Da for LC/ESI/MS and ± 25 ppm for MALDI-FTICR based on instrument accuracy. Masses and their intensities were plotted in SigmaPlot (San Jose, CA) to establish relative signal to noise ratio. The analyses of peptide modifications by HMVK cyclic diadduct 1 (+172.0 Da for LC/ESI/MS and +172.0736 Da for MALDI/FTICR) was included in the analyses for HMVK monoadducts because its mass shift is equivalent to two monoadducts of HMVK. In vivo globin samples were analyzed for all adducts listed above, whereas analyses of the in vitro data was limited to HMVK cyclic diadduct 2 and THB adduct formation since globin and globin peptide HMVK monoadduct formation in vitro was described previously (12).

LC/ESI-MS/MS of Trypsin Digested Fraction

After MALDI-FTICR analyses of HPLC collected peptide fractions from one of the rats treated with 200 mg/kg BDD and its control, the fraction containing the most peptides modified by HMVK monoadducts (20.5-22.5 min) was subjected to a nanoLC-MS/MS fragmentation using 1100 HPLC-MSD SL Ion Trap mass spectrometer (Agilent). Chromatography of peptides was accomplished using a Zorbax (Agilent) C18 stable bond column (3.5 μ m, 0.075 mm $\times$ 150 mm) with the following mobile phases: A, 0.1% formic acid in H₂O and B, 95% ACN, 0.1% formic acid at 280 nL/min, over 60 min with 20% to 80% B gradient. As peptides eluted from the LC/ESI source, MS/MS spectra were collected over 4 channels with a mass range between 300 and 2200 m/z. MS/MS data were then converted to the workable file format to search customized amino acid sequence database using Mascot (Matrix Science; London, UK). The following rat Hb modifications were specified in the search: HMVK monoadducts on unmodified and posttranslationally modified (methionine oxidation and cysteine carbamidomethylation) peptides containing cysteines, lysines, arginines, N-terminal valines and histidines. Although attempts were made at acquiring ms/ms sequencing data using QTOF/MS, these attempts were not successful. Ion Trap/MS proved more sensitive to ions of low abundance (see Results).

Electrospray (ESI)/MS of Intact Globin Chains

Globin from rats dosed with BDD (25 or 200 mg/kg), and saline was subjected to ESI/MS analyses on a Applied Biosystems Sciex API 365 triple-quadrupole electrospray ionization mass spectrometer by direct infusion in a flow path of 50:50 (ACN:H₂O) at 30 μ L/min. The spectra were scanned with a mass range between 400-2200 m/z. Molecular masses of globin chains were obtained by reconstruction of each multiply charged ion spectrum using BioSpec Reconstruct algorithm supplied by Perkin-Elmer BioMultiView, version 1.3.1. The analyses of modified globin was based on searching for mass shifts of the most abundant SD rat globin chains as described above for peptide analyses. The presence of HMVK monoadducts on intact globin chains was determined by a mass shift of (+86 Da), (+172 Da), (+258 Da), and (+344 Da) for addition of 1, 2, 3, and 4 HMVK moieties, respectively. Intact globin was also analyzed for the presence of cyclic diadducts 2 with a mass shift of (+152.0 Da), (+304 Da), (+456 Da),

and (+608 Da) for addition of 1, 2, 3, and 4 HMKV cyclic diadducts 2, respectively. The presence of THB adducts on intact globin chains was determined by a mass shift of (+104 Da), (+208 Da), (+312 Da), and (+416 Da) for 1, 2, 3, and 4 THB adducts, respectively. Parameters to identify adducted chains included: matched masses with an allowable mass error of ± 3 Da, peaks that were above the noise level and absent in control sample.

Results

LC/ESI/MS Analyses of HMKV Monoadducts and/or Cyclic Diadducts 1 on Peptides

The formation of HMKV-Hb adducts was studied by analyzing peptides modified by HMKV monoadducts after rats were dosed with 25 or 200 mg/kg BDD. LC/ESI/MS analyses of trypsin digested globin revealed 8 HMKV modified peptides with both BDD doses (Table 1A). Cys13-containing peptide ($\alpha 1/\alpha 2$ 8-31) with 2 HMKV monoadducts and a Cys125-containing peptide ($\beta 1/\beta 2$ 121-144) with 4 HMKV monoadducts were detected with all rats at both the high and low BDD doses. Since two HMKV monoadducts are equivalent in mass to one cyclic diadduct 1 (+172 Da) (Figure 2), we could not differentiate between the monoadduct or cyclic diadduct 1 presence if the number of HMKV moieties per peptide exceeded one. However, MS analyses of several modified peptides suggested attachment of more HMKV moieties than the number of all nucleophilic sites on these peptides, indicating that these peptides were modified by at least one cyclic diadduct 1. For example, the results presented in Table 1A suggest peptides (1-11) on $\alpha 2$ chain and (31-40) on $\beta 1/\beta 2$ chains have more HMKV moieties than nucleophilic sites (4 HMKV moieties/3 nucleophilic residues and 2 HMKV moieties/1 nucleophilic site on arginine, respectively).

MALDI-FTICR/MS Analyses of HMKV Monoadducts and/or Cyclic Diadducts 1 on Peptides

In order to provide further evidence for HMKV adduct formation on a peptide, globin from a control rat and a rat dosed with 200 mg/kg BDD was trypsin digested and peptide fractions of modified and unmodified globin were collected using HPLC. Concentrated fractions were subjected to MALDI-FTICR/MS and analyzed for matches to theoretical masses of peptides modified by HMKV monoadducts and compared against control. HMKV modified peptides $\alpha 2$ (1-11) and $\beta 1/\beta 2$ (31-40) were detected with both MALDI-FTICR/MS (Table 1B) and LC/ESI/MS (Table 1A). As discussed above the $\beta 1/\beta 2$ (31-40) peptide suggests formation of cyclic diadduct 1 on arginine. In addition, due to higher sensitivity of MALDI-FTICR, the analyses revealed 8 new modified peptides (Table 1B) that were previously not detected by LC/ESI/MS (Table 1A). The fraction collected from 20.5 min to 22.5 min contained the highest number of modified peptides (eight) and suggested formation of cyclic diadducts 1 on lysine of $\beta 1/\beta 2$ (67-76) and on lysine or histidine of $\beta 1/\beta 2$ (133-146). Interestingly, the N- and C-terminal peptides $\alpha 2$ (1-11) and $\beta 1/\beta 2$ (133-146) were detected with one and four HMKV moieties but not with two or three HMKV moieties (Table 1) possibly due to low abundance of these ions and/or due to alternative cross-link formation.

LC/MS/MS Analyses of Peptide Digest Fraction

The HPLC fraction collected from 20.5 to 22.5 min (described above) was subjected to nanoLC/MS/MS analysis using MSD SL Ion Trap mass spectrometer. A Cys125-containing peptide (121-132) on $\beta 1/\beta 2$ chain revealed an ms/ms fragmentation pattern consistent with HMKV monoadduct present at Cys125 (Figure 3). The spectrum of a doubly charged precursor ion (m/z 714.73) displayed 4 b-ions and 8 y-ions. Fragmentation between Thr123 and Pro124 gave rise to a singly charged y_9 -ion (m/z 1049) and a doubly charged y_9 -ion (m/z 525). The high intensity of these ions along with the presence of the corresponding b_3 -ion (m/z 378) confirmed the presence of HMKV on the cysteine-containing fragment of this peptide. In addition, cleavage between Phe122 and Thr123 produced y_{10} ion (m/z 1150) on the modified portion of the peptide. The lower intensity of y_8 ion (m/z 952) cleaved at Cys125 is likely due

to proline's position being adjacent to the cysteine. Fragmentation of the unmodified portion of the peptide gave rise to several expected y- and b- ions confirming identity of the peptide.

In Vivo Formation of HMVK Cyclic Diadduct 2 on Peptides

Characterization of the cyclic diadduct 2 after incubation of HMVK with NAL (14) led us to investigate the formation of such diadducts on amino groups of Hb in vivo (Figure 2) after rats were treated with 25 or 200 mg/kg BDD. Upon trypsin digestion, multiple modified peptides containing HMVK cyclic diadduct 2 were detected by LC/ESI/MS in rats treated with 25 and 200 mg/kg BDD, respectively (Table 2A). Peptides (32-40), (62-90) and (128-141) from $\alpha 1/\alpha 2$ chains and (121-144) from $\beta 1/\beta 2$ chains, containing 1 HMVK cyclic diadduct 2, were present in most rats at both the high and low BDD doses. In addition, all rats dosed with 25 mg/kg BDD revealed peptides (57-60) and (69-99) (α chains) and 62-76 (β chains) modified by HMVK cyclic diadducts 2 (Table 2A). The overlapping sequence between α chain peptides (62-90) and (69-99) suggests that His72, His87, His89, and/or Lys90 may be selective target sites for formation of an HMVK cyclic diadduct 2 at low and high BDD doses.

MALDI-FTICR data of trypsin digested fractions revealed two additional peptides modified by cyclic diadduct 2 in one of the rats dosed with 200 mg/kg BDD (Table 2B). Peptide $\beta 1/\beta 2$ (121-132) described above with an HMVK modification at the cysteine site, was also modified by cyclic diadduct 2 suggesting that both sulfhydryl and amino groups may be involved in reactivity of HMVK with this peptide. The overlap in sequence with modified peptide $\beta 1/\beta 2$ (121-144) detected in all rats at both BDD doses (Table 2A), suggested that Lys132 is consistently involved in reactivity with HMVK.

In Vivo Formation of THB Adducts on Peptides

We have also investigated the formation of EBD in vivo by analyzing formation of THB adducts with globin after rats were treated with 25 or 200 mg/kg BDD. Upon trypsin digestion several peptides modified by THB were detected by LC/ESI/MS at 25 and 200 mg/kg BDD (Table 3A). Peptide $\alpha 1$ (1-7) with 1 THB adduct was detected in all rats at both low and high BDD doses. Interestingly, although LC/ESI/MS revealed modified N-terminal peptides at both high and low BDD doses and majority of the internal peptides were modified at low BDD dose (Table 3A), MALDI/FTICR analyses of digest fractions from a high dosed rat provide additional evidence for the presence of THB adducts on internal peptides (Table 3B). In this regard, peptide $\beta 1/\beta 2$ (66-76) with 1 THB moiety was detected in the whole digest as well as in one of the fractions (Table 3).

In Vitro Formation of HMVK Cyclic Diadduct 2 on Peptides after Exposure of RBCs to HMVK

To corroborate our in vivo results that suggested formation of cyclic diadduct 2 modifications on Hb, we incubated RBCs with HMVK (0.1 and 1 mM) at physiological conditions and subjected whole trypsin digest of globin to LC/ESI/MS analyses. The data revealed the presence of 5 peptides with one or two HMVK cyclic diadducts 2 at high HMVK concentration and one modified peptide at low HMVK concentration (Table 4). As stated above for in vivo experiments, the overlapping sequence between peptide $\alpha 1/\alpha 2$ (69-99) and peptide $\alpha 1/\alpha 2$ (62-90) at low and high HMVK concentrations in vitro, respectively, also suggested that His72, His87, His89, and/or Lys90 may be nucleophilic targets for formation of an HMVK cyclic diadduct 2.

In Vitro Formation of THB Adducts on Peptides after Exposure of RBCs to HMVK

To determine if formation of THB-Hb adducts may be also due to HMVK in addition to EBD (Figure 1), we incubated RBCs with 0.1 and 1 mM HMVK for 2 h at physiological conditions. Analyses of trypsin digested globin from 1 mM HMVK incubation revealed 5 THB adducted

peptides (Table 5) suggesting that HMVK gives rise to THB adducts. Peptide $\beta 1/\beta 2$ (31-59) with 2 THB moieties was detectable at both high and low HMVK concentrations suggesting that it may serve as a selective target for formation of THB adducts from HMVK.

Discussion

In the present study, we have detected HMVK modified peptides after treatment of rats with BDD providing further evidence for both BDD bioactivation to HMVK in rats and for HMVK reactivity with Hb in vivo. HMVK likely forms in the liver via BDD bioactivation by P450s and alcohol dehydrogenases (10,11). The amount that gets translocated into RBCs and binds directly to Hb is presently unknown. Because of HMVK reactivity with sulfhydryl groups (11,14), most of the formed HMVK is likely to conjugate with GSH spontaneously or via GSH transferase in the liver. Significant percentage of the BDD dose (4-8%) was recovered as HMVK-derived mercapturic acids in rats administered BDD providing evidence for initial HMVK-GSH formation (13). Several mechanisms may, however, free HMVK from its adducts for reactivity within RBCs. HMVK-GSH conjugate may undergo a spontaneous or a GSH-transferase-catalyzed retro-Michael reaction as demonstrated previously with the GSH conjugates of trans-4-phenyl-3-buten-2-one and 4-hydroxy-2-nonenal (25,26). Interestingly, the kinetics of HMVK disappearance and HMVK adduct formation in the presence of nucleophilic amino acids suggest the reaction of HMVK with amino groups could be more reversible than the reaction with N-acetyl-L-cysteine since HMVK was not completely depleted and its levels did not change in between 30 min and 6 h after incubation with excess valinamide (14).

All of the HMVK modified peptides that were previously identified in our in vitro study when RBCs were incubated with 0.1 and 1 mM HMVK (12) were also detected after treatment of rats with high and low BDD doses (Table 1). Several Cys13 and Cys125-containing HMVK modified peptides were detected after BDD administration consistent with our previous results that showed higher HMVK reactivity toward NAC over NAL or L-valinamide (14). Peptides $\alpha 1/\alpha 2$ (8-31, containing Cys13) and $\beta 1/\beta 2$ (121-144, containing Cys125) were detected in all rats treated with 25 and 200 mg/kg BDD as well as in vitro when RBCs were incubated with 1 mM HMVK (12). Consistent with the peptide data, ESI/MS of intact globin displayed all chains with 1-4 HMVK moieties at 200 mg/kg BDD (Table S1, Supporting Information). In this regard, Cys125 of rat Hb was shown to be 100 times more reactive than GSH toward 5,5-dithio-bis(2,2-nitrobenzoic acid) (27,28).

To pinpoint specific site of HMVK modification we have chosen trypsin digested fraction 20.5 - 22.5 min collected from one of the two 200 mg/kg BDD treated rats for ms/ms analyses because this fraction contained the most modified Cys-containing peptides. The higher abundance of peptide (121-132) on $\beta 1/\beta 2$ chains enabled successful ms/ms fragmentation which led to identifying Cys125 as the site of modification by HMVK (Figure 3). Although this particular peptide was not present at the lower dose (25 mg/kg BDD), a longer Cys125-containing peptide with a missed cleavage (121-144) was present at both high and low doses (Table 1A), suggesting that Cys125-containing peptides could serve as sensitive biomarkers for BDD/BD exposure. Although attempts were made at acquiring ms/ms sequencing data for other modified peptides, lower abundance of those ions prevented adequate fragmentation. Cys125-containing peptide was likely highly modified by HMVK after in vivo BDD dosing because it is the most reactive site of rat Hb (27,28).

Based on previous characterization of the HMVK cyclic diadducts 1 and 2 with NAL (14), we have investigated in vivo formation of these cyclic diadducts with residues of Hb that contain amino groups. Because cyclic diadduct 1 is equivalent in mass to two monoadducts of HMVK (+172.0 Da), we could not distinguish between these types of HMVK adducts in most peptides

containing several HMVK moieties. However, we did identify 5 unique peptides (Table 1) that exhibited more HMVK moieties than nucleophilic residues suggesting that one or two HMVK molecules could react with the same amino acid to form monoadducts or cyclic diadducts 1. ESI/MS revealed that two of the peptides, (31-40) on β chains and (1-11) on α 2 chains, were present in most rats at the high and low BDD doses implicating Arg40 and Val1, Lys7, or Lys11, respectively, as potential nucleophilic targets for formation of HMVK cyclic diadduct 1.

LC/ESI/MS revealed that the prevalence of peptides modified by cyclic diadduct 2 in vivo was similar to that of peptides modified by HMVK monoadducts/cyclic diadduct 1 (Tables 1A and 2A). This is consistent with the in vitro data that showed NAL monoadduct to readily convert to cyclic diadduct 1 and 2 in the presence of HMVK (14). ESI/MS of intact globin from BDD-treated rats revealed the presence of 2 cyclic diadducts 2 on β 2 chain at both high and low BDD doses in majority of rats (Table S2, Supporting Information) suggesting that cyclic HMVK diadduct 2 also selectively targets β 2 as for HMVK monoadducts. Interestingly, Cys125-containing peptide (121-144) on β chains was detected with both HMVK monoadducts and cyclic diadducts 2 (Tables 1 and 2) in all rats at both the high and low BDD doses suggesting that Cys125-containing peptides could serve as useful biomarkers because they can display formation of different types of HMVK adducts with sulfhydryl and amino groups. Besides lysines, multiple number of cyclic diadducts 2 on the same peptide suggested involvement of other nucleophilic residues such as arginines, N-terminal valines, and histidines. When we incubated RBCs with HMVK (0.1 and 1 mM) at physiological conditions we were able to identify 4 peptides (32-40, 62-90, 69-99 on α chains and 62-76 on β chains) modified by cyclic diadduct 2 (Table 4) that were also detected in vivo (Table 2). Furthermore, the overlapping sequence of α chain peptides (62-90) and (69-99) suggested modifications by cyclic diadduct 2 at His72, His87, His89, and/or Lys90. Peptides (32-40) and (62-90) on α chains were also detectable at both high and low BDD doses in vivo in most rats (Table 2) possibly providing an additional biomarker for HMVK cyclic diadduct 2.

We have also investigated formation of EBD from BDD in vivo by analyzing for THB-Hb adducts after treating rats with BDD. Our data support formation of THB adducts with Hb upon exposure to BDD suggesting formation of EBD in vivo. THB-valine adducts derived from DEB and EBD are currently used for biomonitoring of human exposure to BD (15,19). A modified N-terminal valine-containing peptide α 1 (1-7) was detected at both the high and low BDD doses (Table 3A) consistent with THB-Valine adducts used as biomarkers. Multiple adducts on globin chains and multiple internal peptides indicate that sites of modification other than N-terminal valine may also be involved. THB adducts were previously identified with N-terminal aspartic acid, glutamic acid, histidine, and lysine residues of albumin in vitro when human serum albumin was incubated with EBD at physiological conditions (29).

To determine if formation of THB adducts may be also due to HMVK we have incubated RBCs with 0.1 and 1 mM HMVK for 2 h at physiological conditions. THB adducted peptides were detected at both HMVK concentrations, two of which (α 1 1-7 and α 1/ α 2 69-90; Table 5) were also detected at the low BDD dose (25 mg/kg) in vivo (Table 3A). Consistent with these results, ESI/MS of intact globin revealed THB moieties primarily on α 2 chain in rats treated with both high and low BDD doses (Table S3, Supporting Information). Interestingly, peptide β (31-59) was detectable at both 0.1 and 1 mM HMVK in vitro and peptide β (41-61) with an overlapping sequence was present at both 25 and 200 mg/kg BDD doses in vivo implicating Lys59 as a possible reactive site for THB adducts derived from HMVK. Presence of the above mentioned THB adducts in vitro upon incubation with HMVK and in vivo after BDD dosing suggests that HMVK may contribute to formation of THB adducts in vivo. We propose that HMVK could undergo an initial 1,4-addition in a classical Michael addition reaction with amino residues of Hb followed by enol to keto tautomerization and protonation of the α carbon resulting in an

HMVK monoadduct (Figure 4, pathway a). Alternatively, an initial 1,4-addition can be followed by aziridinium ion formation and hydrolysis (Figure 4, pathway b) resulting in a THB structure identical to the EBD-derived THB structure (Figure 1) carrying the same molecular mass shift of (+104 Da). Thus, HMVK may contribute to THB adduct formation. This finding has toxicological significance because of the likely differences in the toxic properties of HMVK from that of EBD or DEB and the pervasive use of THB adducts for biomonitoring of BD exposure in humans. Involvement of HMVK in THB adduct formation may explain the high levels of these adducts in comparison to adducts resulting from BMO in rats, mice, or humans after BD exposure (30).

In conclusion, we have detected formation of HMVK monoadducts and cyclic diadducts with Hb after BDD treatment demonstrating that HMVK formed from BDD can react with Hb *in vivo*. Contribution of the EBD pathway leading to formation of THB-Hb adducts is not quite as clear because THB adducts may result from both EBD and HMVK. We have demonstrated that HMVK is more likely to alkylate cysteine residues of Hb and pinpointed Cys125 of β chains as a major modification site. Bioactivation of BDD to HMVK could also lead to modification of other cellular proteins leading to formation of monoadducts and/or cyclic diadducts which could play important role in toxicity and/or carcinogenicity of BD. Therefore, further investigation of specific HMVK-Hb adducts as biomarkers of exposure to BD in humans and the toxic effects of HMVK in mammalian cells are warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACN	acetonitrile
BD	1,3-butadiene
BDD	3-butene-1,2-diol
BMO	1,2-epoxy-3-butene
ddH₂O	double deionized water
DEB	1,2:3,4-diepoxybutene
EBD	1,2-dihydroxy-3,4-epoxybutane
ESI-QTOF	Electrospray-Quadrupole Time-Of-Flight
HMVK	hydroxymethylvinyl ketone
MALDI-FTICR	Matrix Assisted Laser-Desorption Ionization/Fourier Transform Ion Cyclotron Resonance
NAC	N-acetyl-L-cysteine
NAL	N-acetyl-L-lysine
RBC	

red blood cell

SD

Sprague-Dawley

TFA

trifluoroacetic acid

THB-Val

N²-(2,3,4-trihydroxybutyl)valine

Figure 1. Scheme of 1,3-butadiene (BD) metabolism. Epoxide hydrolase (EH), Valinamide-hemoglobin (Val-Hb).

Figure 2.
Scheme of HMVK-derived monoadducts and diadducts with Hb.

Figure 3.
LC/MS/MS of peptide $\beta 1/2$ (121-132) modified by HMVK at Cys125.

Figure 4.

Proposed mechanism for formation of THB adducts from HMVK with amino group of Hb lysine. This mechanism may also represent formation of THB adducts from HMVK with N-terminal Valine of Hb.

Table 1

(A) LC/ESI/MS Results of Peptides Modified by HMK Monoadducts (+86.0 Da for 1 HMK, +172.0 Da for 2 HMK, +258.0 Da for 3 HMK, +344.0 Da for 4 HMK) after dosing of rats with BDD (25 and 200 mg/kg). (B) MALDI/FTICR/MS Results of Fractionated Digest Containing Peptides Modified by HMK Monoadducts (+86.0368 Da) as Described Above after Dosing a Rat with 200 mg/kg BDD. Potential Nucleophilic Reactive Sites are Marked in Bold.

(A) LC/ESI/MS						
Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of HMK moieties	n ^{1a} n ^{2b}
α1	(8-31) ^c	TN K NCWG K IGGGHGGEYGEAL Q R	2789.258	2789.518	2	3/3 2/2
β1	(31-40) ^{d, e}	LLVYPWT Q R	1446.726	1446.848	2	3/3 2/2
β1	(121-144) ^d	EFTPCAQA FQ KVVAGVASAL A HK	2788.276	2788.243	4	3/3 2/2
α2	(1-11) ^e	VLSAAD K T N IK	1503.669	1503.930	4	2/3 2/2
α1	(57-61) ^c	A H G K K	626.390	626.411	1	1/3 1/2
α1	(1-7)	VLSADD K	919.339	919.522	2	1/3 1/2
β1	(1-8) ^d	VHLTDA E K	1170.479	1170.657	3	1/3 1/2
β1	(1-30)	VHLTDAE K AAV N GLW G KV N PD D V G GEAL G R	3346.582	3346.146	3	1/3 1/2
(B) MALDI/FTICR/MS						
Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of HMK moieties	Fraction (min)
α2	(1-11)	VLSAAD K T N IK	1245.6687	1245.6381	1	20.5-22.5
β1	(31-40) ^{d, e}	LLVYPWT Q R	1446.7261	1446.6913	2	34.0-40.0
α1	(1-11)	VLSADD K T N IK	1289.6585	1289.6888	1	8.0-10.0
β1	(9-17) ^d	AAV N GLW G K	1001.5052	1001.5182	1	8.0-10.0, 10.0-15.0
β1	(121-132) ^d	EFTPCAQA FQ K	1512.6309	1512.5999	2	18.0-20.5
β1	(121-132) ^d	EFTPCAQA FQ K	1426.6309	1426.6605	1	20.5-22.5
β1	(67-76) ^{d, e}	VINA F NDGL K	1262.5897	1262.5663	2	20.5-22.5
β1	(133-146) ^d	VVAGVASAL A HK Y H	1508.7858	1508.7634	1	20.5-22.5

(A) LC/ESI/MS						
Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of HMVK moieties	n1 ^a n2 ^b
β1	(133-146) ^{d, e}	VVAGVASALAHK YH	1766.7858	1766.8046	4	20.5-22.5
α1	(8-16) ^c	TN I KNCWG K	1149.5359	1149.5481	1	20.5-22.5
α1	(8-16) ^c	TN I KNCWG K	1235.5359	1235.5140	2	20.5-22.5
α1	(61-68) ^c	K VADAL A K	987.4991	987.5196	2	20.5-22.5
α1	(41-56) ^{c, e}	TYF S HIDVSPGSAQ V K	2079.8655	2079.9445	4	28.0-31.0

^a n1= number of rats detected with adduct over total number of rats treated with 25 mg/kg

^b n2= number of rats detected with adduct over total number of rats treated with 200 mg/kg

^c peptides α1 (8-16), (8-31), (61-68), (41-56), and (57-61) could also be from α2

^d peptides β1 (1-8), (9-17), (31-40), (67-76), (121-132), (121-144), and (133-146) could also be from β2

^e peptides modified by at least one HMVK cyclic diadduct 1 (+172.0 Da for LC/MS and +172.0736 Da for FTICR, equivalent to 2 HMVK moieties)

(A) LC/ESI/MS Results of Peptides Modified by HMVK Cyclic Diadduct 2 (+152.0 Da for 1 diadduct, +304.0 Da for 2 diadducts, +456.0 Da for 3 diadducts, and +608.0 Da for 4 diadducts) after Dosing of Rats with BDD (25 and 200 mg/kg). (B) MALDI/FTICR/MS Results of Fractionated Digest Containing Peptides Modified by HMVK Cyclic Diadduct 2 (+152.0473 Da) as Described Above after Dosing a Rat with 200 mg/kg BDD. Potential Nucleophilic Reactive Sites for Diadduct 2 are Marked in Bold.

(A) LC/ESI/MS						
Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of diadducts 2	n1 ^a n2 ^b
α1	(62-90) ^c	VADALAKA ADH VEDLPGALSTLS DLHA HK	3117.586	3117.881	1	3/3 2/2
α1	(128-141) ^c	FLASVSTVLT SKYR	1723.927	1723.817	1	3/3 2/2
α1	(32-40) ^c	MFAAFPT TK	1165.560	1165.780	1	2/3 2/2
β1	(121-144) ^d	EFTPCAQA AFQ KVVAGVASA LHK	2596.323	2596.621	1	3/3 2/2
α1	(57-60) ^c	A HGK	546.047	546.326	1	3/3 1/2
α1	(69-99) ^c	A ADH VEDLPGALSTLS DLHA HKLRVDPV NFK	3821.903	3822.043	3	3/3 1/2
β1	(62-76) ^d	A HGK KKVINAFNDGL K	1763.947	1764.158	1	3/3 1/2
α1	(12-16) ^c	NCW GK	759.314	759.696	1	1/3 2/2
(B) MALDI/FTICR/MS						
Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of diadducts 2	Fraction (min)
β1	(121-132) ^d	EFTPCAQA AFQ K	1492.6782	1492.7052	1	15.0-18.0
β1	(96-104) ^d	LHVD PENR	1430.6946	1430.7126	2	34.0-40.0

^a n1= number of rats detected with adduct over total number of rats treated with 25 mg/kg
^b n2= number of rats detected with adduct over total number of rats treated with 200 mg/kg
^c peptides α1 (12-16), (32-40), (57-60), (62-90), (69-99), and (128-141) could also be from α2
^d peptides β1 (62-76), (96-104), (121-132), and (121-144) could also be from β2

Table 3

(A) LC/ESI/MS Results of Peptides Modified by THB Adducts (+104.0 Da for 1 THB, +208.0 Da for 2 THB, +312.0 Da for 3 THB, and +416.0 Da for 4 THB) after Dosing of Rats with BDD (25 and 200 mg/kg). (B) MALDI/FTICR/MS Results of Fractionated Digest Containing Peptides Modified by THB Adducts (+104.0473 Da) as Described Above after Dosing a Rat with 200 mg/kg BDD. Potential Nucleophilic Reactive Sites For THB adducts are Marked in Bold.

(A) LC/ESI/MS						
Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of THB Moieties	n ^b
α1	(1-7)	VLSADDK	851.436	851.443	1	3/3
β1	(41-61) ^c	YFDSFGDLSSASAIMGNPKVK	2442.195	2441.850	2	3/3
β1	(77-95) ^c	HLDNLKGTFAHLSELHCDK	2594.266	2593.819	4	3/3
β1	(66-76) ^c	KVINAFNDGLK	1322.732	1322.808	1	2/3
α1	(61-68) ^d	KVADALAK	1023.594	1023.659	2	2/3
α1	(69-90) ^d	AADHVEDLPGALSTLSDLHAHK	2609.295	2609.516	3	2/3
β1	(1-17) ^c	VHLTDAEKAAVNGLWGK	2225.155	2225.471	4	0/3
(B) MALDI/FTICR/MS						
Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of THB Moieties	Fraction (min)
β1	(66-76) ^c	KVINAFNDGLK	1322.7319	1322.7391	1	34.0-40.0
α1	(128-141) ^d	FLASVSTVLTSKYR	1675.9270	1675.9154	1	15.0-18.0
β1	(1-8)	VHLTDAEK	1016.5473	1016.5476	1	20.5-22.5
β1	(62-66) ^c	AHGKK	644.3731	644.3800	1	25.5-28.0
β1	(60-65) ^c	VKAHGK	743.4473	743.4301	1	23.5-25.5

^a n1= number of rats detected with adduct over total number of rats treated with 25 mg/kg
^b n2= number of rats detected with adduct over total number of rats treated with 200 mg/kg
^c peptides β1 (1-17), (41-61), (60-65), (62-66), (66-76), (77-95), and (133-146) could also be from β2

d peptides $\alpha 1$ (61-68), (69-90), and (128-141) could also be from $\alpha 2$

LC/ESI/MS Results of Peptides Modified by HMOVK Cyclic Diadduct 2 after Incubating Rat RBCs with HMOVK for 2h at pH 7.4, 37°C. Potential Nucleophilic Reactive Sites for Cyclic Diadduct 2 are Marked in Bold.

Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of diadducts 2
1 mM HMOVK					
α1	(32-40) ^a	MFAAFTTK	1165.560	1165.733	1
α1	(62-90) ^a	VADALAKAAD H VEDLPGALSTLS D L H A H K	3269.624	3269.275	2
β1	(1-17) ^b	V HLTDAEKAAV N GLWG K	2113.061	2113.140	2
β1	(62-76) ^b	A H G K KVINAFNDGL K	1763.947	1763.976	1
β1	(105-132) ^b	LLGNMIVIVL G HH L GKEFTPCAAAFQ K	3339.727	3339.681	2
0.1 mM HMOVK					
α1	(69-99) ^a	AAD H VEDLPGALSTLS D L H A H K L RVD P V N F K	3821.903	3821.466	3

^a peptides α1 (32-40), (62-90), and (69-99) could also be from α2

^b peptides β1 (1-17), (9-40), (62-76), and (105-132) could also be from β2

Table 5

LC/ESI/MS Results of Peptides Modified by THB Adducts after Incubating Rat RBCs with HMVK for 2h at pH 7.4, 37°C. Potential Nucleophilic Reactive Sites are Marked in Bold.

Hb chain	Peptide	Sequence	Mass _{theo} (Da)	Mass _{obs} (Da)	# of THB moieties
1 mM HMVK					
α1	1-7	VLSADDK	955.484	955.551	2
α1	(12-40) ^a	NCWGKGGGGGEEALQRMFAAFPTTK	3467.625	3467.772	3
α1	(69-90) ^a	AADHVEDLPGALSTLSDL HAHK	2609.295	2609.507	3
α1	(91-99) ^a	LRVDPVNF K	1295.721	1295.726	2
β1	(31-59) ^b	LLVVYPWTQRYFDSFGDLSSASAIMGN PK	3470.695	3470.958	2
0.1 mM HMVK					
α1	(61-68) ^a	KVADALAK	1023.594	1023.5	2
β1	(31-59) ^b	LLVVYPWTQRYFDSFGDLSSASAIMGN PK	3470.695	3470.7	2

^a peptides α1 (12-40), (61-68), (69-90), and (91-99) could also be from α2

^b peptide β1 (31-59) could also be from β2