Diazotization of kynurenine by acidified nitrite secreted from indoleamine 2,3-dioxygenase-expressing myeloid dendritic cells

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Abbreviations: BMDC, bone marrow-derived myeloid dendritic cells; CpG, CpG oligodeoxynucleotides; DC, dendritic cells; IDO, indoleamine 2,3-dioxygenase; Kyn, kynurenine; NMA, N^G-monomethyl-L-arginine acetate; NO, nitric oxide; NOS, NO synthase; TCA, trichloroacetic acid; Trp, tryptophan

Abstract

Indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan metabolism along the kynurenine (Kyn) pathway regulates T-cell responses in some dendritic cells (DC) such as plasmacytoid DC. A Kyn assay using HPLC showed that samples were frequently deproteinized with trichloroacetic acid (TCA). In the present study, bone marrow derived myeloid DC (BMDC) were differentiated from mouse bone marrow cells with GM-CSF. CpG oligodeoxynucleotides (CpG) induced the expression of IDO protein with NO production in BMDC cultured for 24 hr. The concentrations of Kyn in the culture supernatants were not increased by stimulation with CpG but rather decreased by based on the Kyn assay after deproteinization with TCA. The level of Kyn exogenously added into the cell-free culture supernatant of BMDC stimulated with CpG was severely decreased by deproteinization with TCA but not methanol, and the decrease was prevented when BMDC was stimulated with CpG in the presence of a NOS inhibitor. Under acidic conditions, Kyn reacted with nitrite produced by BMDC, and generated a new compound that was not detected by Ehrlich reagent reacting with the aromatic amino residue of Kyn. An analysis by mass spectrometry showed the new compound to be a diazotization form of Kyn. In conclusion, the deproteinization of samples by acidic treatment should be avoided for the Kyn assay when NO is produced.

Key words: Bone marrow-derived myeloid dendritic cells; Indoleamine 2,3-dioxygenase; Tryptophan; Kynurenine; Nitric oxide; Diazotization

1. Introduction

The indoleamine 2,3-dioxygenase (IDO)-initiated tryptophan (Trp) metabolism along the kynurenine (Kyn) pathway regulates the T-cell responses in some dendritic cells (DC) such as plasmacytoid DC or $CD8^+$ DC in mouse spleen cells (Mellor and Munn, 2004). IDO is induced by inflammation or immune responses such as infections or tumor immunity. IDO expression is induced in DC by various stimuli such as IFN-γ, toll-like receptor (TLR)-ligation by LPS or CpG oligodeoxynucleotides (CpG) or CD80/CD86-ligation by CTLA-4 expressed on the regulatory T cells (Mellor and Munn, 2004). The induction of IDO activity was observed in IFN-γ-activated murine peritoneal macrophages when nitric oxide synthase (NOS) was inhibited (Thomas *et al*., 1994). Incorporation of the heme prosthetic group into the active site is required for IDO activity, and the inhibition of IDO activity by NO generators was abrogated by co-addition of oxyhemoglobin, an antagonist of NO function (Thomas *et al*., 1994). NO also led to an accelerated degradation of IDO protein in the proteasome (Hucke *et al*., 2004).

To determine IDO activity, three assay methods are commonly used. They are based on the conversion of L-Trp to *N*-formyl-kynurenine followed by hydrolysis to produce Kyn. The amount of Kyn produced can be quantified directly by HPLC or through radiometric methods. Alternatively, a colorimetric method is used to measure Kyn indirectly. This technique relies on absorption of the imine produced by the reaction of the aromatic amino group of Kyn with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) at λ max 480 nm (Takikawa *et al*., 1988). Formerly, Kyn was measured by diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine (Mason and Berg, 1952). For diazotization, Kyn was treated with sodium nitrite in trichroloacetic acid (TCA) solution. IDO activity has been frequently estimated by the Kyn assay using samples deproteinized by acidic treatment before HPLC assay

(Takikawa *et al*., 1988; Fujigaki *et al*., 2002; López *et al*., 2006). Nitrite is a stable derivative of NO. It has therefore been suggested that acidic treatment induces an underestimation of Kyn when NO is produced.

In the present study, Kyn was degraded by treatment with TCA for deproteinization through the reaction with nitrite produced by BMDC stimulated with CpG. The errors were avoided by the Kyn assay using methanol for deproteinization.

2. Materials and methods

2.1. Reagents

The phosphorothioate CpG1826 (5-TCC ATG ACG TTC CTG ACG TT-3), N^G-monomethyl-L-arginine acetate salt (NMA), and L-Kyn were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse IDO polyclonal antibody was prepared as described previously (Suzuki *et al*., 2001). Anti-inducible NOS (iNOS) polyclonal antibody was purchased from BD Bioscience (San Diego, CA). Human serum was purchased from Gemini Bio-Product (Woodland, CA)

2.2 Preparation of BMDC

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All experiments were done according to the Guidelines for Animal Experimentation Nagoya University. BMDC were generated as described previously (Inaba *et al*., 1992). Briefly, bone marrow cells were cultured in RPMI1640 medium (10% fetal calf serum, 300 μg/ml glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol) containing 0.3% GM-CSF supernatant (from murine GM-CSF producing Chinese hamster ovary cells, a gift from T. Sudo, Toray Silicon, Tokyo,

Japan). The DC culture medium was changed every 2 days to remove any nonadherent cells. Loosely adherent clustering cells were collected on day 6 and then were used as immature DC. BMDC were activated by stimulation with CpG (5 μg/ml) alone or together with NMA (100 μM) for 24 hr.

2.3. Characterization BMDC by Flow cytometry, Western blot and NO production

For the detection of cell surface markers, cells were incubated with PE-conjugated anti-CD11c and FITC-conjugated anti-CD11b antibodies at 4℃ for 30 min. These cells were analyzed by an EPICS XL flow cytometer (Beckman Coulter).

A Western blot analysis was carried out as described previously (Du *et al*., 2000). BMDC were stimulated with CpG (5 μg/ml) with or without NMA (100 μM) for 24 hr. The cell lysates were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels by electrophoresis and then transferred to a nitrocellulose membrane. The membrane was incubated with anti-IDO, anti-iNOS or anti-actin antibody at 4℃ overnight, then with horseradish peroxidase-conjugated anti-rabbit IgG antibody for 1 hr at room temperature, and finally, developed with a Western lightning chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA).

The amount of NO production in the medium was estimated by the assay of nitrite using Griess reagent (Ding *et al*., 1988). Fifty microliter of each supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water). The absorbance of the mixture at 590 nm was determined by a plate reader, and nitrite concentration was determined using standard solutions of sodium nitrite.

2.4. Assay for Kyn

Concentrations of Kyn were determined by HPLC as previously described (Takikawa *et al*., 1988; Widner *et al*., 1997), with minor modifications. Before HPLC assay, the culture medium was deproteinized by the treatment with 5% TCA or 86% methanol (1:6, vol/vol). Twenty-five microliters of sample was injected into a 5 μm endcapped Purospher RP-18 column (Merck, Darmstadt, Germany) and analyses were carried out at a flow rate 1.0 ml/min. The mobile phase was 10 mM acetic ammonium (pH 6.5) containing 10% methanol (Takikawa *et al*., 1988) or 15 mM acetic acid-sodium acetate (pH 4.0) containing 27 ml/l acetonitrile (Widner *et al*., 1997). Kyn was detected by UV-detector at a wavelength of 360 nm.

Kyn was also assayed indirectly by using Ehrlich reagent (0.4% *p*-dimethylaminobenzaldehyde in acetic acid; Takikawa *et al*., 1988). Samples treated or untreated with 5% TCA were centrifuged, and 100 μl supernatant was mixed with 100 μl Ehrlich reagent. The absorbance at 490 nm was determined.

2.5. UV visible spectroscopy and Mass spectrometry

The spectra were recorded at room temperature on a UV-vis-NIR recording spectrophotometer (Shimadzu, Kyoto, Japan).

Mass spectroscopy for Kyn and its derivatives was carried out using a LCQ Deca XP (Thermo Fisher Scientific, CA, USA), which consisted of an electrospray and a 3D ion trap detector, by direct infusion of the samples. The conditions for the MS analysis were as follows: ion spray voltage, 2.5 kV; ion transfer tube, 250℃; scan range, 150 - 500 m/z; picotip, 15 μm IDCE; collision energy, 35%.

3. Results

3.1. NO-linked secretion of TCA-triggered Kyn-degrading substances from BMDC

stimulated with CpG

BMDC were differentiated with GM-CSF for 6 days. Most (67.1%) of the BMDC were $CD11c^+CD11b^+$ myeloid DC (**Fig. 1A**). The expression of IDO and iNOS proteins was induced in BMDC stimulated with CpG for 24 hr (**Fig. 1B**). NO production was also induced in BMDC stimulated with CpG (**Fig. 1C**). These results show that the expression of IDO protein and iNOS activity is induced in BMDC by stimulation with CpG.

The production of Kyn by the IDO^+ BMDC was examined in the culture supernatants by HPLC after deproteinization with TCA. The level of Kyn in the culture supernatant of IDO^+ BMDC was not increased even after the exogenous addition of Trp and rather decreased by the stimulation with CpG (data not shown). The concentration of exogenously added Kyn to the culture of BMDC markedly decreased by CpG stimulation and this decrease of Kyn concentration was partially inhibited by NMA (data not shown). These results suggest the possibility that NO-related products of BMDC degrade Kyn. The Kyn-degrading activity of the culture supernatants of BMDC was tested. Kyn (50 μM) was added for 2 hr into the cell-free culture supernatant of BMDC stimulated with CpG in the presence or absence of NMA for 24 hr. Because TCA and methanol have been used for deproteinization, both methods were tested comparatively. The Kyn concentration (50 μM) in the TCA-treated culture supernatants of BMDC stimulated with CpG was decreased by 50% and this decrease was prevented by NMA (**Fig. 1D**). In contrast, the Kyn concentration of the culture supernatant of BMDC stimulated with CpG was not decreased by methanol treatment. These results indicate that methanol treatment for deproteinization is suitable for the measurement of Kyn in order to avoid TCA-triggered Kyn degradation. The effects of pH of elution buffer in the HPLC assay on Kyn degradation were also tested. The Kyn concentrations estimated by HPLC using an elution buffer at pH 4.0 and pH 6.5 were

almost identical. These results indicate that elution buffer at higher pH than pH 4.0 is suitable for avoiding Kyn degradation by acidic treatment. The HPLC elution profile of the absorption at 360 nm was shown in **Figure 1E**. The treatment of the culture supernatant of BMDC stimulated with CpG with TCA yielded a new absorption peak at a shorter retention time (3.9 min), corresponding to the decrease of the absorption peak of Kyn at a retention time (6.3 min).

3.2. Reaction of Kyn with nitrite under acidic conditions detected by HPLC, Ehrlich reagent and absorption spectra

The amount of Kyn-degrading substance secreted from BMDC was correlated with NO production. Therefore, the effects of nitrite, a stable NO derivative, on the Kyn treated with TCA were tested by HPLC. The absorption of 50 μM Kyn in PBS at 360 nm was completely lost by TCA-treatment in the presence but not absence of 50 μM sodium nitrite (**Fig. 2A**). Corresponding to the disappearance of the absorption peak of Kyn, a new absorption peak appeared at a shorter retention time (**Fig. 2B**). This change of the absorption of Kyn was the same with that observed by TCA-treatment of the mixture of Kyn and the cell-free culture supernatant of BMDC stimulated with CpG (**Fig. 1E**). These results indicate that in response to the TCA treatment, Kyn reacts with nitrite secreted from BMDC stimulated with CpG. The extent to which TCA-treatment influences Kyn measurement was further studies in human serum. As shown in **Figure 2C**, the concentration of Kyn (50 μM) exogenously added into human serum was decreased by more than 20% by treatment with 0.5% TCA in the presence of 50 μM $NaNO_2$. This concentration of TCA was 10 times lower than that required for deproteinization.

The concentration of Kyn was also indirectly assayed using Ehrlich reagent which reacts with the amino residue of the benzoic ring of Kyn (Takikawa *et al*., 1988).

Ehrlich reagent could not detect any compounds generated through the TCA-triggered reaction of Kyn with nitrite, however, reacted with TCA-treated Kyn (**Fig. 2D**). These results suggest that the amino residue of the benzoic ring of Kyn is used for the TCA-triggered generation of a new compound, and Ehrlich reagent does not react with the altered Kyn.

The pH-dependence and kinetics of the reaction of Kyn with nitrite in PBS were assayed by absorption spectroscopy. The absorption peak of Kyn at 360 nm was completely lost by the TCA treatment in the presence of 50 μM $NaNO_2$, and a new absorption peak at 340 nm appeared (**Fig. 2E**). This change in the absorption was induced by treatment with nitrite at pH 1-4 (**Fig. 2F**). The absorption peak of Kyn at 360 nm was rapidly lost by the treatment with TCA in the presence of $NaNO_2$, whereas the new absorption peak at 340 nm increased gradually after 60 min (**Fig. 2G**). On the other hand, the absorption peak of Kyn at 360 nm was decreased by 90% without the appearance of a new absorption peak by the treatment with TCA alone (**Fig. 2E**). The reversibility of the reaction of Kyn with nitrite by acidic treatment was tested (**Fig. 2H**). The absorption spectrum of a new compound generated through the reaction of Kyn with nitrite by treatment with HCl at pH 1 was not reversed by treatment at neutral pH. However, neutral pH treatment completely recovered the acid-triggered loss of the absorption spectrum of Kyn within a second. These results indicate that the reaction of Kyn with nitrite by acidic treatment is irreversible, whereas the change in the absorption of Kyn induced by acidic treatment is reversible.

3.3. Mass spectrometric analysis of the diazo compound of Kyn through the reaction with $NaNO_2$ under acidic conditions

The new compound generated by the reaction of Kyn with nitrite under acidic conditions was analyzed by mass spectrometry. A proton adduct of Kyn $[M+H]^+$

appeared at 209 m/z (**Fig. 3A**). This peak was lost by the treatment of Kyn with TCA (**Fig. 3B**) or HCl at pH 1 (**Fig. 3C**) in the presence of $NaNO_2$, and new peaks at 220 m/z and 182 m/z appeared as main products. The product with $[M]^+$=220 m/z shows an increase mass of 12 Da from mass of Kyn (208 Da). This difference in molecular mass corresponds to the generation of a new compound of Kyn through the addition of nitrogen atom of $NaNO_2$ (+N:+14) to an amino group at the aromatic ring of Kyn with releasing 2 hydrogen atoms (-2H:-2). This suggests that a diazo ($-N^+\equiv N$) compound from an amino residue of the benzoic ring of Kyn was derived by the reaction of Kyn with $NaNO_2$ under acidic conditions (**Fig. 3D**). The possibility of the formation of a diazocompound at α-amino group is excluded, since the diazo group formed from primary amine is very unstable. Furthermore, lack of reactivity of Ehrlich reagent with the generated compound (**Fig. 2C**) also supports formation of the speculated compound. The product at 182 m/z was strongly retained in C18 column of HPLC, thus showing a different polarity from Kyn derivatives (data not shown). Furthermore, the depletion of molecular group corresponding to 26 m/z from Kyn is hardly estimated. Although it is difficult to speculate on the structure of the product at present, it might not have originated from Kyn.

4. Discussion

TCA treatment for deproteinization has been frequently done before the Kyn assay by HPLC. The current study showed that TCA treatment induced errors in the estimation of the Kyn assay by acid-triggered reaction of Kyn with nitrite produced by BMDC stimulated with CpG. This error could be avoided by deproteinization with methanol. It has been shown that NO production inhibits IDO at the post-transcriptional (Thomas *et al*., 1994; Hucke *et al*., 2004) or transcriptional (Alberati-Giani *et al*., 1997) levels. The present study suggests that the IDO activity is

underestimated unless acidic treatment is avoided in the Kyn assay, when NO is produced.

We demonstrated the reaction of Kyn with nitrite in TCA solution by the assay of HPLC, absorption spectra and mass spectrometry. The data of the absorption spectra shows that the reaction of Kyn with acidified nitrite is induced irreversibly and gradually for 1 hr at a lower pH than pH 4. These results are consistent with the report that the diazonium salt of Kyn was decomposed, apparently completely, by incubation for an hour at 36℃ (Mason and Berg, 1952). The loss of the absorption spectrum of Kyn by TCA-treatment alone is very rapid and reversible so this change of Kyn absorption is not detected by HPLC assay using elution buffer at pH 4.0 - 6.5.

The product generated by the reaction of Kyn with acidified nitrite could not be detected by Ehrlich reagent that reacts with the amino residue of the aromatic ring (Takikawa *et al*., 1988), thus suggesting the modification of the aromatic amine of Kyn by acidified nitrite. The data of mass spectrometry suggest the generation of a new compound of Kyn through the reaction of the nitrogen of $NaNO_2$ with one of 2 amino residues of Kyn. Based on the above findings, we conclude that the diazotization of Kyn is generated through the reaction with acidified nitrite secreted from BMDC stimulated with CpG. Under acidic conditions, protonation of nitrite ions yield nitrous acid (HNO_2), which can be protonated a second time. Water leaves the molecule forming the nitrosonium ion (+NO), which acts as an excellent nucleophile to modify a number of amino acids and secondary amines. Griess described the diazotization of aryl amine by nitrite and coupling of the product to form an azochrome in 1879 (Griess, 1879). The diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine was applied for the Kyn measurement (Mason and Berg, 1952). However, these reports were published when the biological action of NO production was not known. The current study showed for the first time the errors in the estimation of the Kyn assay caused by the acid-initiated diazotization of Kyn in the biological system producing

NO.

Some reports show which concentrations of Kyn produced in the NO-generating systems have been assayed by HPLC after deproteinization by acidic treatment (Fujigaki *et al*., 2002; López *et al*., 2006). *Toxoplasma gondi* infection increase the levels of both Kyn and NO in the plasma of mouse, and a NOS inhibitor enhance the Kyn concentration (Fujigaki *et al*., 2002). IFN-γ-induced increase of the concentration of Kyn in the culture supernatant of human monocytic U-937 cells is inhibited by a NO donor (López *et al*., 2006). The present study suggests the possibility that the IDO activities in these reports may be underestimated by the diazotization of Kyn induced by acidified nitrite.

In conclusion, the deproteinization of samples by acidic treatment should therefore be avoided for the Kyn assay when NO is produced.

Acknowledgements

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Figure Legends

Figure 1. NO-linked secretion of TCA-triggered Kyn degrading substance from BMDC stimulated with CpG. (A) Surface expression of CD11c and CD11b on BMDC was analyzed by flow cytometry. The percentage of cells in each quadrant is presented. (B) IDO and iNOS expressions were assessed by a Western blot analysis. (C) Nitrite accumulation in the culture supernatant was measured using Griess reagent. Means ± SD of triplicate cultures are presented. (D, E) After BMDC (3×10^5 cells/0.2 ml) were stimulated with CpG alone or together with NMA for 24 hr, Kyn (50 μM) were added to the cell-free culture supernatants and incubated at 37℃ for 2 hr. Then, culture supernatants were deproteinized with 5% TCA or 86% methanol, and concentrations of Kyn were measured by HPLC using acetic acid-sodium acetate buffer (pH 4.0) or acetic ammonium buffer (pH 6.5). (D) The means ± SD of triplicate cultures are presented. (E) HPLC elution (pH 4.0) profiles of the absorption at 360 nm with the retention time (min) of the culture supernatants treated with 5% TCA for deproteinization were presented.

Figure 2. The reaction of Kyn with nitrite under acidic conditions detected by HPLC, Ehrlich reagent and absorption spectra. (A-D) Kyn (50 μM) were added with or without $NaNO_2$ (50 μM) in (A, B, D) PBS or (C) human serum. (A, B, D) These solutions or (C) human serum 2-fold diluted with PBS was incubated with or without (A, B, D) 5% or (C) the indicated % of TCA at 37℃ for 2 hr. Concentrations of Kyn in the reaction solution were assayed by HPLC with elution buffer at (A, B) pH 4.0 or (C) pH 6.5 after deproteinization with 86% methanol at neural pH, or (D) using Ehrlich reagent. (A, C, D) Means ± SD of triplicate cultures and (B) HPLC elution profiles of the absorption at 360 nm with retention time (min) are presented. (E, F) Kyn (50 μM) in PBS was incubated with 50 μM $NaNO_2$ together with or without (E) 5% TCA or (F)

HCl at the indicated pH at 37℃ for 2 hr. (G) Kyn was incubated with $NaNO_2$ and TCA at 37℃ for 0-2 hr. (H) Kyn was incubated with HCl alone at pH 1 or together with $NaNO_2$ at 37℃ for 2 hr. Thereafter, the pH of these reaction solutions was adjusted to pH 7, and the volume of reaction solution was adjusted. The absorption spectra of these samples are presented.

Figure 3. The analysis of compounds of Kyn generated through the reaction with nitrite under acidic conditions by mass spectrometry. (A) Kyn (50 μM) in water was incubated with 50 μM $NaNO_2$ together with (B) 5% TCA or (C) HCl at pH 1 at 37℃ for 2 hr, and dried with methanol. These samples were analyzed by mass spectrometry. (D) The predictable structure of a diazo compound generated by the reaction of Kyn with $NaNO_2$ under acidic conditions.

Answers to reviewers' comments:

Reviewers' comments:

Reviewer #1: This article presents evidence to explain underestimated concentrations of kynurenines in samples pretreated with TCA for deproteinization prior to HPLC analysis. The authors show that CpG treated BMDC express IDO and INOS. The nitrite present in cell culture supernatants reacts with the TCA and produces a diazotized form of kynurenine that is not detected at 360 nm as is normal kynurenine. This result was supported by absorbance curves showing the existence of a new compound that absorbed at 340 nm and by mass spec data showing increased mass consistent with the formation of a diazo group. The use of MeOH as a deproteinizer did not produce the diazo-kynurenine product nor did TCA pre-treatment of samples from experiments which used NMA a NO inhibitor. The authors conclude that TCA pre-treatment of samples for Kyn analysis by HPLC should be avoided, particularly in cases where NO is produced or just using MeOH to deproteinize.

This technical study explains potential underestimation of Kyn concentrations reported in the literature, and provides an alternative method of avoiding this technical problem by using a different reagent. Though no important scientific results are presented current interest in the IDO field elevates this report to some significance, especially as the authors performed background experiments with mass spectroscopy and studied the effects of pH on diazo-formation. In summary, this report should be published as a technical article.

The article would benefit by being read by a native English speaker to clarify some of the points the authors try to make to connect the claims to the supporting data. In several places, the text is rather jumbled, and clear connections between IDO, NO expression by CpG treated cells and TCA pre-treatment of HPLC samples leading to an underestimation of kynurenines are not evident. Thus, though data is convincing it was difficult to follow the authors' interpretations.

Answer: A native English speaker has carefully revised the manuscript.

Reviewer #2: This is an interesting paper, which stresses an important issue. The findings are certainly especially relevant for studies in mice and rats. To be more

complete, it would be important to know to what extent TCA-treatment might influence kynurenine measurements in human sera/plasma of healthy individuals and patients with inflammatory conditions.

Answer: Kynurenine measurements in human sera/plasma of healthy individuals and patients are very difficult, because clinical doctors were not involved in this study. Besides, it is hard to resolve this issue within the short time given to revise this manuscript, because clinical work takes long time. Therefore, we studied the extent to which TCA-treatment influences Kyn measurements in human serum. As shown in Fig. 2C, the concentration of Kyn (50 μM) exogenously added into human serum was decreased by more than 20% by treatment with 0.5% TCA in the presence of 50 μM $NaNO_2$. This concentration of TCA was 10 times lower than that required for deproteinization. We described these results in Fig. 2C.

Figure 1

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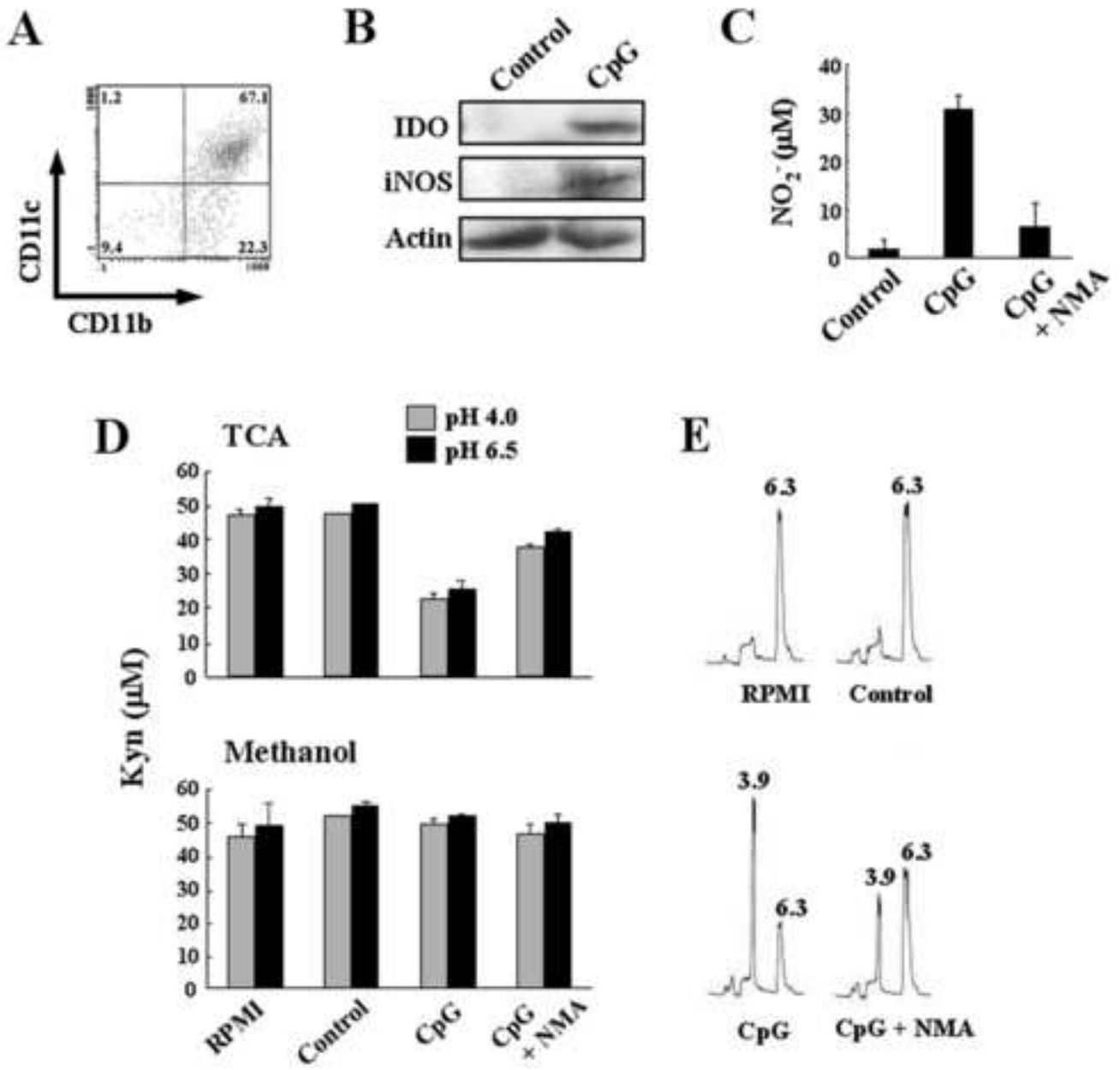

Figure 2

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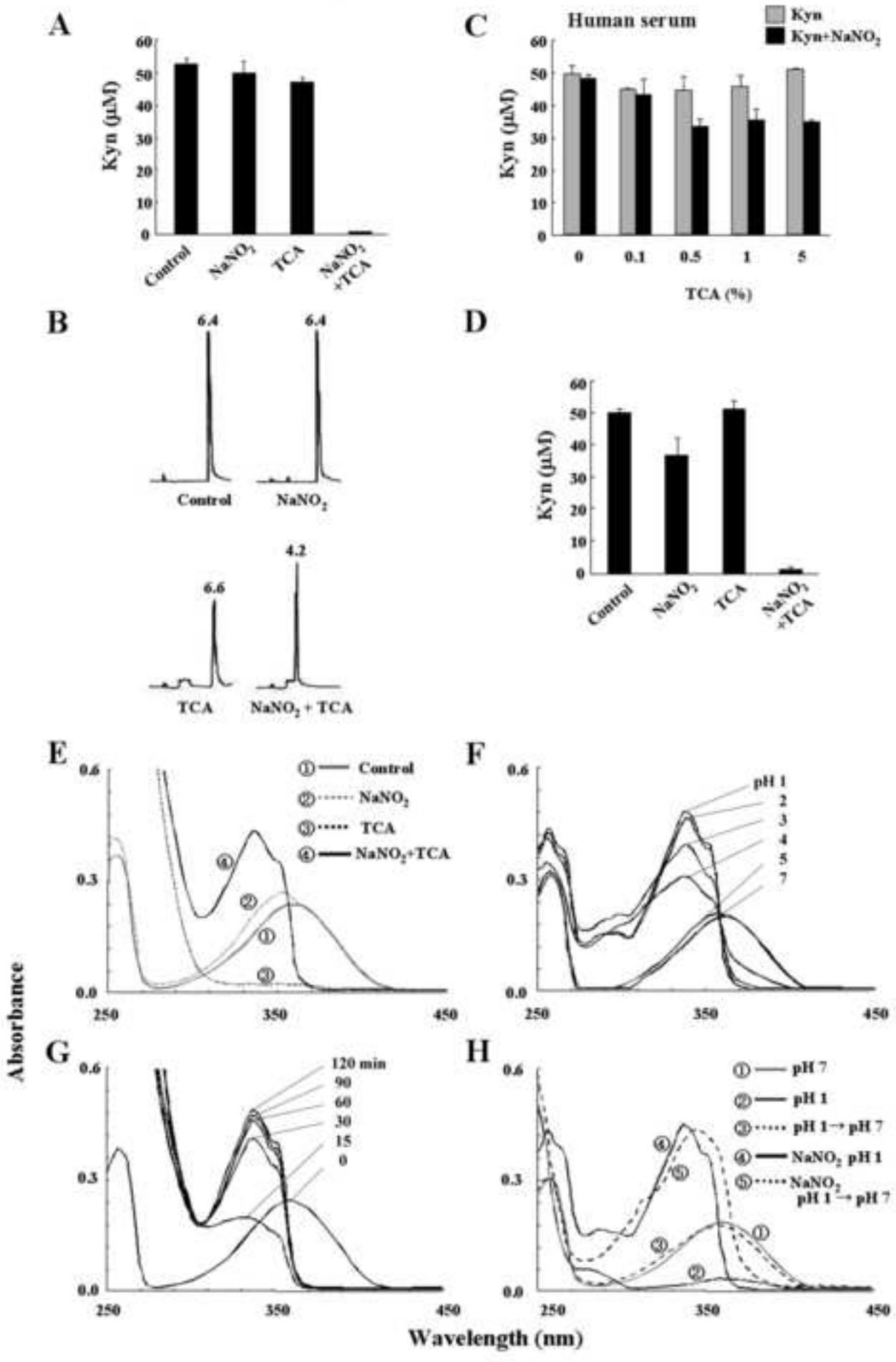

Figure 3

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