Palmitate deranges erythropoietin production via transcription factor ATF4 activation of unfolded protein response

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Lipotoxicity plays an important role in the progression of chronic kidney damage via various mechanisms, such as endoplasmic reticulum stress. Several studies proposed renal lipotoxicity in glomerular and tubular cells but the effect of lipid on renal erythropoietin (EPO)-producing (REP) cells in the interstitium has not been elucidated. Since renal anemia is caused by derangement of EPO production in REP cells, we evaluated the effect of palmitate, a representative long-chain saturated fatty acid, on EPO production and the endoplasmic reticulum stress pathway. EPO production was suppressed by palmitate (palmitate-conjugated bovine serum albumin [BSA]) or a high palmitate diet, but not oleic acid-conjugated BSA or a high oleic acid diet, especially under cobalt-induced pseudo-hypoxia both *in vitro* and *in vivo*. Importantly, suppression of EPO production was not induced by a decrease in transcription factor HIF activity, while it was significantly associated with endoplasmic reticulum stress, particularly transcription factor ATF4 activation, which suppresses 3′-enhancer activity of the EPO gene. ATF4 knockdown by siRNA significantly attenuated the suppressive effect of palmitate on EPO production. Studies utilizing inherited super-anemic mice (ISAM) mated with EPO-Cre mice (ISAM-REC mice) for lineage-labeling of REP cells showed that ATF4 activation by palmitate suppressed EPO production in REP cells. Laser capture microdissection confirmed ATF4 activation in the interstitial area of ISAM-REC mice treated with palmitate-conjugated BSA. Thus, endoplasmic reticulum stress induced by palmitate suppressed EPO expression by REP cells in a manner independent of HIF activation. The link between endoplasmic reticulum stress, dyslipidemia, and hypoxia may contribute to development and progression of anemia in CKD.

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Accumulating evidence demonstrates that dyslipidemia is pathophysiologically significant in chronic kidney disease (CKD). The lipid nephrotoxicity theory was first proposed by Moorhead *et al.*[1] in 1982. Clinically, CKD is often associated with lipoprotein abnormalities, and such patients show a more rapid decrease in kidney function.[1–4] Further, dyslipidemia due to lifestyle accelerates the progression of CKD.[5] Under an excessive or imbalanced intake of dietary fat, long-chain saturated fatty acids are more harmful than unsaturated fatty acids.[6] Excessive saturated fatty acids are esterified and accumulated as intracellular lipid droplets in several organs, including the liver, pancreas, skeletal muscle, arterial wall, and adipose tissue, and thereby induce various cellular dysfunctions associated with abnormal lipid metabolism. In fact, the consumption of palmitate, a representative long-chain saturated fatty acid, has markedly increased with recent growth in Western food habits. Excessive intake of palmitate induces pathogenic stress signals, including endoplasmic reticulum (ER) stress, oxidative stress, and systemic inflammation in various cells,[7] which in turn exacerbate various disease phenotypes, including obesity, insulin resistance, type 2 diabetes, nonalcoholic fatty liver disease, atherosclerosis, and cardiovascular disease, as well as CKD.[7] To date, however, the molecular mechanism of palmitate-induced nephrotoxicity has not been completely understood.

In this study, we focused on the effect of palmitate on anemia in CKD. Renal anemia is associated with a poor prognosis in patients with CKD.[8,9] Renal anemia is mainly caused by defective production of erythropoietin (EPO), a glycoprotein hormone that maintains erythropoiesis. EPO production is strictly regulated by the hypoxia inducible factor (HIF) pathway. The HIF machinery is deranged under certain pathogenic conditions, however, such as inflammation and oxidative stress, leading to renal anemia.[10] Our previous studies showed that indoxyl sulfate, a representative uremic toxin, induces malfunction of renal EPO-producing (REP) cells and suppresses EPO production.[11,12]

Here, we investigated whether the long-chain saturated fatty acid palmitate is involved in the suppression of EPO production. We found that palmitate suppressed EPO production via induction of the unfolded protein response (UPR), an ER stress signal, in renal EPO-producing cells, and in particular that it activated the UPR transcriptional factor ATF4.

RESULTS

Palmitate suppressed EPO production in HepG2

To assess the effect of palmitate on EPO production, the hepatic EPO-producing cell line (HepG2) was treated with different doses of palmitate-conjugated bovine serum albumin (PAL-BSA) or fatty acid–free BSA (referred to as BSA) as a control under the normoxic or hypoxic conditions. On treatment with up to 120 μM PAL-BSA or BSA for 24 hours, the basal level of EPO mRNA expression was not changed during the experimental period (Figure 1a). Interestingly, 120 μM PAL-BSA, but not BSA, significantly suppressed EPO mRNA production only when EPO production was stimulated by hypoxia (1% O_2) or pseudo-hypoxia (a pharmacological activator of HIF $CoCl_2$, 100 μM) (Figure 1b). These phenomena were associated with intracellular lipid droplet accumulation (Supplementary Figure S1). Such lipid droplet accumulation was not associated with the alteration of the master regulators involved in β-oxidation (peroxisome proliferator-activated receptor α), fatty acid uptake (cluster of differentiation 36), or mitochondrial biogenesis (PGC1α) (Supplementary Figure S2), suggesting that the incorporation of palmitate into the cells affected the EPO production machinery without affecting mitochondrial lipid metabolism.

Palmitate selectively impaired hypoxia-induced EPO production without affecting HIF activity in HepG2

EPO production is strictly regulated by the HIF pathway, an O_2-sensing machinery. To evaluate whether PAL-BSA suppressed EPO production by affecting HIF activity, we performed Western blot analysis for detection of nuclear accumulation of HIF. Although hypoxia-induced EPO mRNA expression was significantly suppressed by PAL-BSA in HepG2 (Figure 1), nuclear accumulation of HIF, especially HIF-2α, which is known as the transcriptional factor of EPO production, was not changed by PAL-BSA or BSA under pseudo-hypoxia with $CoCl_2$ (Figure 2a). These data were consistent with a lack of change in the mRNA expression level of other representative HIF target genes, such as adrenomedullin and heme oxygenase-1, by PAL-BSA (Figure 2b). These data indicate that PAL-BSA selectively interfered with EPO mRNA expression without affecting HIF activity.

Suppression of EPO production by palmitate was associated with ER stress, especially ATF4 activation, in HepG2

Previously, we demonstrated that EPO production was suppressed by an ER stress inducer that activates the UPR pathway.[13] We therefore hypothesized that the suppression of EPO mRNA by PAL-BSA is mediated by UPR activation. As

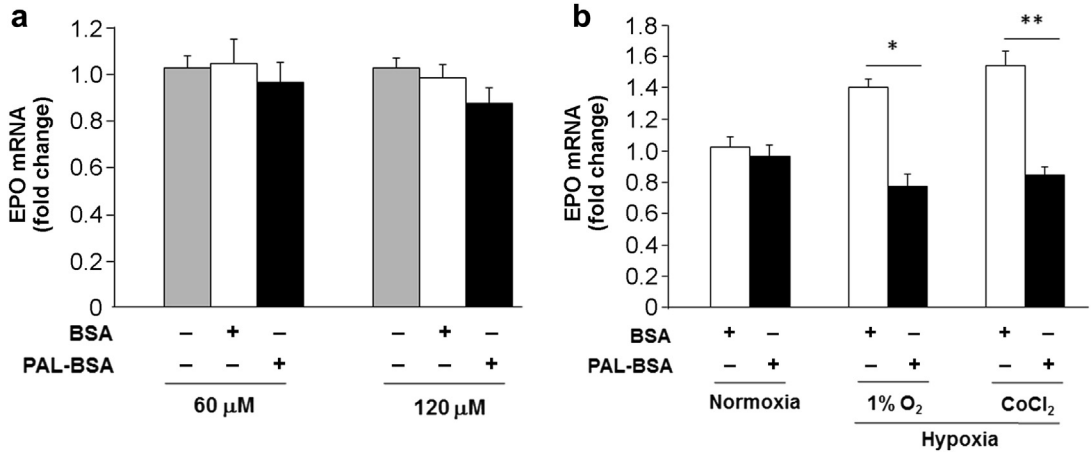

Figure 1 | Effect of palmitate on EPO expression. (**a**) Palmitate did not change the basal level of erythropoietin (EPO) mRNA expression. HepG2 was treated with various concentration of PAL-BSA or BSA under normoxic conditions for 24 hours, and then EPO mRNA expression was measured by real-time polymerase chain reaction (PCR). EPO mRNA expression was not changed by palmitate-conjugated bovine serum albumin (PAL-BSA) or BSA under normoxic conditions. (**b**) Palmitate suppressed EPO mRNA production when EPO production was stimulated by hypoxia (1% O_2) or pseudo-hypoxia (100 μM $CoCl_2$). HepG2 was treated with 120 μM of PAL-BSA or BSA for 12 hours, followed by exposure to hypoxia or normoxia for 12 hours. EPO mRNA expression was then measured by real-time PCR. Each group was subject to 2 or 3 assays, which were repeated 3 or 4 times. *$P < 0.05$, **$P < 0.01$. Plus and minus signs indicate addition and without addition, respectively.